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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/68, 33/577, C12P 21/08, C12N 5/18, C07K 7/08, 7/10 — A1

(11) International Publication Number: WO 94/14070

(43) International Publication Date: 23 June 1994 (23.06.94)

(21) International Application Number: PCT/CA93/00522

(22) International Filing Date: 6 December 1993 (06.12.93)

(30) Priority Data:
984,123 4 December 1992 (04.12.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 984,123 (CIP)
Filed on 4 December 1992 (04.12.92)

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: IMMUNOASSAY FOR THE MEASUREMENT OF COLLAGEN CLEAVAGE IN CARTILAGE

(57) Abstract

A method for determining the degradation of cartilage in a biological sample is described. The method involves measuring the amount of unwound collagen present in a biological sample through the binding of unwound collagen with a monoclonal antibody. The monoclonal antibody selected for use in the method has the ability to bind to an epitope on unwound collagen chains or fragments of collagen containing this epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen. The present invention also relates to a kit for the measurement of cartilage degradation.

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TITLE OF THE INVENTION

Immunoassay for the Measurement of Collagen Cleavage in Cartilage.

FIELD OF THE INVENTION

The invention relates to a method for evaluating cartilage degradation by determining the level of collagen cleavage products in a biological sample. In a preferred embodiment, the invention relates to an immunoassay for measuring type II collagen cleavage in cartilage.

BACKGROUND OF THE INVENTION

The physiological turnover of articular cartilage represents a fine balance between synthesis and degradation. It is a feature of normal growth and development and maintenance of cartilage in the adult. Net cartilage destruction with ensuing loss of joint function is a feature of the arthritides. An understanding of the factors mediating cartilage breakdown and its control is therefore of great importance, since from this an approach can be made to design therapies for the reduction of pathological cartilage destruction and the enhancement of repair in this tissue. Progress to this goal is dependent on identifying the degradative events occurring in articular cartilage and correlating these with the many degradative agents potentially active in the tissue.

Type II collagen constitutes the bulk of the fibrillar backbone of cartilage matrix, just as type I collagen forms the fibrillar organization of the extracellular matrix of most other tissues such as skin, bone, ligaments and tendons. These collagens are composed of a tightly wound triple helix, which can only be cleaved by the metalloproteinase collagenase to produce 3/4 and 1/4 length α-chain fragments that are identifiable by polyacrylamide gel electrophoresis.

The destruction of articular cartilage is due, in part, to the degradation of the extracellular matrix, which is composed primarily of fibrillar type II collagen and aggregating proteoglycans. In articular cartilage, type II

collagen fibrils are responsible for the tensile strength whereas the proteoglycans provide the compressive stiffness necessary for normal articulation and function. The precise mechanisms by which these connective tissue components are degraded are not fully understood. In mammals, one mechanism involves collagenase, an enzyme capable of a site-specific cleavage of helical (native) collagen.

Incapable of maintaining a helical structure at physiological temperatures, collagenase-cleaved collagens unwind and become susceptible to further degradation by other proteinases in the extracellular space. In this regard, collagenase can be considered the rate limiting enzyme involved in collagen degradation. A variety of studies have provided indirect evidence that proteolytic enzymes are implicated in the destruction of articular cartilage.

Dodge and Poole (1) provided direct immunohistochemical evidence that type II collagen was degraded in situ. This was accomplished by developing a rabbit polyclonal antibody to unwound collagen residues.

Limited proteolytic degradation of collagen in cartilage has been demonstrated to result in a loss of the tensile strength of cartilage with negligible release of collagen from the cartilage matrix, measured biochemically.

Traditional methods for the detection of collagen loss have relied on the use of stains such as van Gieson's. The latter was used by Fell and her collaborators (2) to detect collagen loss in cartilage: this results in a loss of the normal bright-pink staining. This method, like others of its kind, is, however, of unproven specificity.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for the quantitative determination of cartilage degradation. The method generally comprises the measurement of the amount of unwound collagen present in a biological sample through the binding of unwound collagen with a monoclonal antibody. The monoclonal antibody

selected for use in the method has the ability to bind to an epitope on unwound collagen chains or fragments of collagen containing this epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen.

The method of the present invention can further include a preliminary step in which the biological sample is contacted with an enzyme having the ability to selectively cleave unwound collagen chains without degrading the epitope recognized by the monoclonal antibody. This can be done to selectively extract proteolytically denatured collagen domains which might be unavailable for antibody binding because of their retention in the biological sample, due to cross-linking or fibrilar packing. Accordingly, this step is useful when the biological sample is a biological tissue or fluid.

A further step by which the biological sample is treated to solubilize and unwind remaining native collagen it contains without degrading the epitope recognized by the monoclonal antibody can also form part of the method of the present invention. This allows the measurement of the degree of unwinding of collagen in cartilage, as the binding of the monoclonal antibody to the unwound collagen chains can be quantitatively compared to the binding of the antibody with the remaining native collagen which has been solubilized and unwound. However, in order to accurately quantitate the degree of unwinding, the amount of wound collagen that is extracted by the above described enzyme (that selectively cleaves unwound collagen) should also be measured. This is done by treating an aliquot of the enzyme extract to solubilize and unwind any native collagen contained therein.

The present invention also provides a method for the determination of cartilage degradation by quantitating the amount of unwound collagen, said method comprising:

- contacting a biological sample with an enzyme having the ability to selectively cleave unwound

collagen chains in said biological sample into collagen fragments without cleaving an antibody-reactive epitope on said unwound collagen chains;

- extracting the collagen fragments that react with said enzyme from said biological sample to produce an enzyme extract;
- removing an aliquot from said extract and treating said aliquot to unwind any wound collagen contained therein;
- treating said biological sample to solubilize and unwind remaining native collagen contained therein without degrading the antibody reactive epitope;
- measuring the amount of unwound collagen present in said extract, said aliquot of said extract and said solubilized biological sample by separately contacting said extract, said aliquot and said biological sample with a monoclonal antibody which has the ability to bind an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen, and determining the amount of said unwound collagen and fragments thereof bound to said monoclonal antibody; and
- determining the amount of collagen that is unwound by comparing the immunological reaction of said monoclonal antibody to said extract, said aliquot and said solubilized biological sample.

The invention also relates to a method for measuring total collagen content in a biological sample. The method comprises treating the biological sample to solubilize and unwind collagen without degrading the epitope recognized by the antibody. The amount of unwound collagen and fragments thereof present in the solubilized biological sample is then measured by contacting the biological sample with a monoclonal antibody which has the ability to bind an epitope on unwound collagen chains or fragments thereof containing the epitope. The monoclonal

antibody does not bind to native helical collagen. The amount of unwound collagen and fragments thereof bound to the monoclonal antibody is then determined.

The present invention also relates to a monoclonal antibody which has the ability to bind to an epitope on unwound collagen chains or fragments of unwound collagen chains containing this epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen. The invention also includes a cell line producing this monoclonal antibody.

Also in accordance with the present invention is a kit for the measurement of cartilage degradation products in a biological sample. The kit comprises a monoclonal antibody which has the ability to bind to an epitope on unwound collagen chains or fragments thereof containing the epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen. The kit also comprises a solid support for binding proteins and a labelled antibody to measure the binding of the monoclonal antibody to the unwound collagen.

The invention also includes an immunogenic synthetic peptide sequence. This sequence is characterized in that it has a sufficient number of residues to allow for sufficient antibody activity when used as an immunogen. It comprises at least a portion of a hydrophilic domain of peptide CB11 of type II collagen α-chains and it does not include hydroxylysine residues.

Also within the scope of the present invention is a method for the in vivo detection of collagen damage in cartilage found in joints. The method involves injecting a patient with a radiolabeled monoclonal antibody or fragment thereof having the ability to bind to an epitope on unwound collagen chain or fragments of collagen containing this epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen. The method then involves measuring the in vivo binding of the

antibody to exposed collagen epitopes in joint cartilage by means for detecting the label on the antibody.

The invention represents the first available assay for the specific detection of cartilage type II/XI degradation products in hyaline cartilage and body fluids. This technology may also be used for the establishment of an assay for the detection of type I and other collagen degradation products.

For the first time, suitable antibody reactive epitopes on the type II collagen chain have been identified. Also, enzymatic techniques are provided by which collagen and fragments of collagen can be solubilized and extracted from a biological sample to allow an accurate determination of collagen breakdown in fluids and tissues. These fragments and unwound collagens can be detected *in situ* in cartilage and in serum and joint fluids in various animals and humans. Since this is the first assay of its kind, it has considerable potential value.

Hence, with the present invention, the degree of unwinding of type II/XI collagen in cartilage or other tissues can be measured quantitatively for the first time. Since it is possible to measure fragments containing an antibody-reactive epitope in joint fluid and serum/plasma, these cartilage specific type II/XI collagen fragments can be detected and quantitated in animals, patients and healthy persons. Thus, the assay has potential for monitoring cartilage collagen degradation in children with impaired growth and in adults with rheumatoid arthritis and osteoarthritis to measure abnormal cartilage collagen degradation in patients with osteoporosis and to determine effects of therapy *in vivo* and its effects on cartilage turnover at the degradative level. The invention can also be used to measure collagen degradation in intervertebral discs. Also, the invention has much potential in studying effects of new drugs on cartilage metabolism in experimental animals in drug development by pharmaceutical companies.

The present invention will be more readily illustrated by referring to the following description.

IN THE DRAWINGS

Figure 1 represents the complete amino acid sequence (SEQ ID NO:4) of type II collagen. The peptide fragments obtained through cyanogen bromide degradation of the triple helical region are identified as CNBr peptides 1-15. Abbreviations are as follows: P., peptide; Trip. Hel., triple helix; C.Link., cross link; STR, stromelysin cleavage site; PEP, pepsin cleavage site. Note that collagenase cleaves the triple helix at a glycine-leucine bond which is represented in the figure by residues 906 and 907 (underlined).

Figure 2 represents the hydrophobicity/hydrophilicity plot for the CB11 peptide of type II collagen. A negative hydrophobicity value represents a hydrophilic region of the amino acid sequence.

Figure 3 represents the amino acid sequences of A. synthetic peptide CB11B (SEQ ID NO:3) and B. synthetic peptide CB11B/H (SEQ ID NO:4). The latter represents the epitope in CB11B that is seen by monoclonal antibody COL2-3/4m.

Figure 4 represents SDS-PAGE Coomassie brilliant blue stained bovine collagens (panel a) and Western immunoblot analysis of the same collagens for reactivity with non-immune ascitic fluid (panel b) or ascitic fluid containing antibody COL2- 3/4m (panel c). In each panel, lane 1 is type I collagen; lane 2 is type II collagen; lane 3 is type III collagen; lane 4 is medium enriched for type X collagen; lane 5 is type XI collagen. Arrows show the positions of the α3 chain of type XI collagen; α1 chain of type II collagen; type X collagen.

Figure 5 represents SDS-PAGE Coomassie brilliant blue stained bovine type II collagen after cleavage by collagenase (panel a) and Western immunoblot analysis of the same collagenase- cleaved collagen for reactivity with monoclonal COL2-3/4m (panel b). Arrows indicate the

positions of the intact α1(II) chains as well as the 3/4 and 1/4 products that are characteristic of collagenase cleavage.

Figure 6 represents the standard curve of the percentage of inhibition against the concentration of peptide CB11B in an inhibition ELISA.

Figure 7 represents A. µg/ml or B. molar concentration/response profiles for inhibition in an ELISA assay by heat denatured collagen (HDC) compared with peptide CB11B.

Figure 8 represents a comparison of the concentration of CB11B and hydroxyproline in different HDC samples.

$r = 0.9984$ ($p<0.0001$)

Figure 9 represents a comparison between the percentage inhibition by heat denatured collagen (HDC) and native collagen (NC) detected by the method of the present invention.

Figure 10 represents the relative concentrations of the CB11B epitope in sera of normal children and adults and in children with chondrodysplasias.

Figure 11 represents the relative concentrations of the CB11B epitope in sera from normal adults and patients with osteoporosis, rheumatoid arthritis (RA) and osteoarthritis (OA).

Figure 12 represents the relative concentration of the CB11B epitope in synovial fluid (SF) and paired serum from individual patients with rheumatoid arthritis. Each line represents one patient.

Figure 13 represents the relative concentrations of the CB11B epitope in patients with rheumatoid arthritis (RA), osteoarthritis (OA) or in normal groups in sera and synovial fluids (SF).

Figure 14 represents results of immunoassays of normal human serum (NHS) or serum from rheumatoid arthritis (RA) and osteoarthritis (OA) patients. The percentage inhibition of COL2- 3/4m antibody binding by each serum is

shown to exhibit parallelity to percentage inhibition produced by the CB11B peptide.

Figure 15 represents the percentage denatured collagen in bovine foetal epiphyseal cartilage discs.

Figure 16 represents the analysis of type II collagen denaturation (unwinding) in human femoral condylar articular cartilage from non-arthritic (normal) and osteo-arthritic (OA) patients. Each bar represents one patient.

Figure 17 represent SDS-PAGE and Western immuno-blot analysis of bovine type II collagen cleaved by human collagenase or cyanogen bromide. A) Coomassie brilliant blue stained 7.5% SDS-PAGE gel (panel a) and Western immunoblot with antibody COL2-3/4m (panel b) of type II collagen following cleavage by human recombinant collagen-ase. Arrows indicate uncleaved α-chain and the 3/4 and 1/4 cleavage products. B) Silver-stained 12% SDS-PAGE gel (panel a) and Western immunoblot with a control ascitic fluid (see legend to Fig. 2) (panel b) or ascitic fluid containing antibody COL2-3/4m (panel c) of cyanogen bromide peptides of type II collagen. Arrows indicate the major cyanogen bromide peptides. Staining with coomassie or silver-stain and the western immunoblots were performed as described in methods.

Figure 18 represents total and denatured type II collagen in human femoral condylar cartilage from different patient groups. Each point is the result for cartilage obtained from one patient. Total collagen is shown as µg/mg wet weight and denatured collagen is expressed as a % of total collagen in each specimen. Bars indicate the mean value for each patient group. Statistical analysis is by Mann-Whitney U-test.

Figure 19 represents lack of any correlation between Mankin grade and total or denatured type II collagen in OA cartilage. Total collagen is shown as µg/mg wet weight and denatured collagen is expressed as a % of total collagen in each specimen.

Figure 20 represents the variation in total and denatured type II collagen with depth in normal and OA human femoral condylar cartilage. Plugs of cartilage were divided into the more superficial zone (upper 1mm) and deeper zone (lower 1mm) using a scalpel. Each line connects upper and lower cartilages obtained from one patient. Total collagen is shown as µg/mg wet weight and denatured collagen is expressed as a % of total collagen in each specimen. Individual patients are identified by the numbers N1-N5 for normal cartilage and OA1-OA8 for OA cartilage. A comparison of the mean values for each patient group is shown in Table VII. Statistical analysis is by Mann-Whitney U-test.

Figure 21 represents a schematic representation of adult human L5-S1 intervertebral disc. The wedge-shaped stippled area was removed at autopsy and 6 blocks of tissue (3 AF and 3 NP) were taken from the sampling sites marked x.

Figure 22 represents the content of total type II collagen, shown as µg/mg wet weight of tissue, in cartilage (lined bars), AF (stippled bars) and NP (black bars) from 7 autopsy cases, A-G. The mean values and SD for each tissue type are also shown. *$p<0.01$ v. cartilage (2-tailed paired t-test).

Figure 23 represents the content of hydroxyproline, shown as µg/mg wet weight of tissue, in cartilage (lined bars), AF (stippled bars) and NP (black bars) from 7 autopsy cases, A-G. The mean values and SD for each tissue type are also shown. *$p<0.01$ v. cartilage (2-tailed paired t-test).

Figure 24 represents the ratio of µg total type II collagen/µg hydroxyproline in cartilage (lined bars), AF (stippled bars) and NP (black bars) from 7 autopsy cases, A-G. The mean values and SD for each tissue type are also shown. *$p<0.01$ and NS=not significant v. cartilage (2-tailed paired t-test).

Figure 25 represents the content of % denatured type II collagen in cartilage (lined bars), AF (stippled bars) and NP (black bars) from 7 autopsy cases, A-G. The mean values and SD for each tissue type are also shown. *p<0.01 v. cartilage (2-tailed paired t-test).

Figure 26 represents the content of proteoglycan, shown as µg GAG/mg wet weight of tissue, in cartilage (lined bars), AF (stippled bars) and NP (black bars) from 7 autopsy cases, A-G. The mean values and SD for each tissue type are also shown. *p<0.01 and NS=not significant v. cartilage (2-tailed paired t-test).

Figure 27 represents the correlation of type II collagen with GAG concentration in cartilage (panel a; r=0.41, NS), AF (panel b; r=0.68, NS) and NP (panel c; r=0.78, p<0.05) from 7 autopsy cases.

Figure 28 represents immunohistochemical identification of type II collagen denaturation in cartilage (A), AF (B) and NP (C) using monoclonal antibody COL2-3/4m. Controls were sections of AF stained with non-immune ascitic fluid (D) or COL2-3/4m pre-absorbed with peptide α1(II)-CB11B (E).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a quantitative immunoassay for the measurement collagen cleavage. Examples of collagen that can be measured include type I, II, III, IV, VI, IX and XI collagen. The assay is particularly useful to determine the degradation of type I or type II/XI collagen.

For type I collagen, the assay of the invention is useful to detect and measure type I collagen fragments in serum. Bone and skin contain type I collagen which is excessively degraded in bone in osteoporosis. This degradation of type I collagen leads to an increased release of collagen cross-links which at present can only be detected in urine. The assay of the invention is sufficiently sensitive to study sera of patients suffering from skin diseases and osteoporosis. It represents an improvement

over currently available immunoassays for collagen degradation which are not sufficiently sensitive for serum assays.

For type II collagen, the assay is useful to detect and measure type II collagen fragments in serum, synovial fluids and cartilage. Type II collagen fibrils form the skeletal framework of hyaline cartilages and provide it with its tensile strength. The assay represents the first quantitative method to detect the degradation of cartilage through the analysis of type II collagen breakdown.

The assay is constituted by providing a monoclonal antibody to a synthetic peptide derived from a collagen segment that is specific to the collagen to be measured. In the examples provided later to illustrate rather than limit the scope of the invention, a synthetic peptide produced on the basis of the sequence of human type II collagen α-chains has been prepared. The antibody reacts with both this peptide and the three identical α-chains found in the human, bovine, rat, hamster, rabbit, mouse and horse. It also recognizes type II collagen α-chain fragments which contain this epitope. It does not however react with native triple helical type II collagen but only with unwound α-chains or fragments thereof containing the epitope. To the extent possible, the peptide chosen to prepare the antibody is specific to the collagen to be evaluated. However, the antibody prepared can cross-react with a minor component of cartilage collagen fibrils showing the same structure, namely type XI collagen α_3 chain without affecting the overall accuracy of the assay. The epitope (CB11 B/H) which is recognized by the antibody, is not present in any other protein, according to protein sequence analyses.

The antibody is used to develop an enzyme-linked immunoassay to detect unwinding of helical collagen when it is degraded *in situ*. It will be appreciated that the present invention is not restricted to any specific type of collagen, although the preferred assay embodiments that

have been developed are specific to type II collagen. This antibody can be used for quantitative assay, for immunohistochemistry and for isolation of type II collagen α-chains or fragments thereof containing the peptide sequence recognized by the antibody. Also, by radiolabeling the monoclonal antibody of the invention, it can be used for clinical imaging of sites of cartilage degradation.

In situations where the biological sample to be assayed is a tissue rather than a body fluid, it is desirable to pretreat the sample to provide a collagen extract that allows for optimal binding of the unwound collagen with the antibody. For example, application of the protease chymotrypsin to cartilage results in the selective cleavage of unwound type II collagen. The fragments obtained are released from unwound type II collagen without digesting the epitope that is recognized by the antibody. Thus, unwound degraded collagen can be detected *in situ*. Furthermore, remaining helical collagen can be solubilized and unwound without digesting the epitope that is recognized by the antibody, preferably through the action of an enzyme such as proteinase K, and measured by being reacted with the same antibody. Each of the constituents used in the method of the present invention is described in further detail below.

Preparation of immunogenic peptides

In order to raise antibodies that can be efficiently used in the method of the present invention, it is necessary to determine from which portion of the overall collagen chain synthetic peptides having suitable immunogenic properties can be prepared. In one of the preferred embodiments of the invention, efforts were concentrated on a specific region of the type II collagen α-chain identified as the CB11 region, named after peptide 11 of the cyanogen bromide degradation method described by Scott et al. (3) hereby incorporated by reference. Sites on the CB11 fragment of type II collagen that have hydrophilic

domains were studied further for immunogenic properties. It was determined that criteria for the selection of a suitable peptide sequence are as follows:

a) the peptide should not include any hydroxylysine residues (these were likely to be substituted with carbohydrate chains, or could involve cross-links, that could block antibody-binding);

b) the peptide sequence should be well-conserved between species (to permit species cross-reactivity); and

c) the peptide sequence should have minimal homology with sequence from the α-chains of other collagens besides type II.

Once a given portion of the collagen chain has been identified, based on the criteria set forth above as being suspected of possessing the desired immunogenic properties, the appropriate immunogenic sequence can then be chemically synthesized using standard chemistry and equipment such as the Applied Biosystems Model 431A solid phase peptide synthesizer. Once the appropriate peptide has been prepared, it can be coupled to an immunogenic vehicle, such as ovalbumin, to immunize mice from which monoclonal antibodies can be prepared. The resulting antibodies are then evaluated for reactivity to the peptide and heat deratived collagen (HDC) to determine whether they can be used to establish an inhibition assay.

In a preferred embodiment of the present invention, a synthetic peptide was prepared consisting of a 21 amino acid sequence from the CB11 peptide of human type II collagen. The 21 amino acid sequence was synthesized with an additional N-terminal cysteine and a C-terminal tyrosine, resulting in a 23 amino acid peptide (termed CB11B, SEQ ID NO:3) of the formula:

C-G-K-V-G-P-S-G-A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-Q-Y

In the above formula, (OH) represents hydroxylation of proline, C is used to bind to ovalbumin and Y can be used for labeling with ^{125}I if required for radioimmunoassay applications.

In the case of this peptide, it was assumed that all proline residues in the "Y" position of the Gly-X-Y repeat sequence were hydroxylated. However, the hydroxyl group does not appear to contribute significantly to the hydrophilicity of the overall CB11 peptide since the hydrophilicity profile was almost identical when hydroxyproline values were compared to those of proline.

Monoclonal antibodies to synthetic peptides and preparation of cell lines

As mentioned previously, the monoclonal antibody to be used in the context of the present invention has the ability to bind to an epitope on unwound collagen chains or fragments of collagen chains containing this epitope. The monoclonal antibody is also characterized in that it does not bind to native helical collagen.

The antibodies are prepared by immunizing animals with a peptide having the characteristics referred to above, conjugated to an appropriate immunogen. A preferred peptide for immunization is the above described CB11B peptide coupled to ovalbumin. The techniques employed to prepare the monoclonal antibodies are standard techniques which are known to those skilled in the art. One of the preferred hybridoma cell lines that was prepared is further described in the examples. It has been deposited at the American Type Culture Collection under accession number HB 11202. This cell line produces a monoclonal antibody (termed COL2-3/4m) that has the ability to bind to an epitope on unwound type II collagen chains or fragments containing the epitope. The monoclonal antibody does not bind to native helical type II collagen and cross-reacts with the α_3-chain of type XI collagen, which has the same structure as the α_1(II) chain of type II collagen but is a minor component of cartilage representing about 1-2% of the total type II collagen.

The minimal size of the epitope recognized by the monoclonal antibody COL2-3/4m of the invention is 13

residues. The sequence of this epitope (termed CB11B/H, SEQ ID NO:4) is as follows:

CB11B/H A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-

Alternatively, monoclonal antibodies which recognize heat denatured type II collagen, the immunizing peptide and one sub-peptide can be used.

The monoclonal antibodies that are to be used in the present invention are tested for inhibition with heat denatured type II collagen and the peptide used for immunization. Good inhibition would tend to indicate that the immunoassay can be used as such without further modification. The selective binding of the monoclonal antibody to the peptide used in immunization or native unwound collagen is important in order to have an assay that will be sufficiently sensitive to allow quantitative determination of cartilage breakdown. Cross-reactive antibodies are to be avoided if possible and this is where the selection of the synthetic peptide used in the production of the antibodies is important.

With the preferred antibodies prepared in the context of the present invention, it was demonstrated that with the synthetic peptide on an ELISA plate, antibody binding could be inhibited with the peptide. However, if denatured type II collagen α-chains were bound on the plate, a more sensitive assay was achieved upon inhibition with the synthetic peptide. With heat-denatured type II collagen provided on the ELISA plate, the synthetic peptide and HDC (heat denatured collagen) produced similar inhibition on a molar basis. The antibody did not react with native helical type II collagen.

<u>Proteases for cleaving unwound collagen and for unwinding native helical collagen</u>

When type II collagen is cleaved in the helical region by enzymes such as mammalian collagenase, it unwinds, exposing α-chains that can be cleaved by a variety of proteinases including trypsin and chymotrypsin, which ordinarily cannot cleave the helical regions of native

collagen. This concept was used to selectively extract proteolytically denatured helical type II collagen domains which might be retained in assayed tissue samples by cross-linking and fibrilar packing. Proteases can also be used to cleave unwound collagen chains present in biological fluids to enhance the overall sensitivity of the assay procedure but their use in this context is optional.

It is important to select a protease that will not cleave or degrade a region of the epitope that is reactive to the monoclonal antibody used in the assay. This can be achieved by either preparing an antibody that binds to an epitope not cleaved by the desired enzyme or by selecting an enzyme that cleaves collagen in a region outside the targeted epitope. In the context of the present invention, chymotrypsin is one preferred enzyme that can be used to cleave unwound collagen chains. Another suitable enzyme is trypsin. Unsuitable enzymes include papain and pepsin.

In order to assess the level of unwinding of collagen in a biological sample, it is necessary to measure the total amount of native helical collagen present in the analyzed sample. If the amount of native helical collagen is to be determined through the use of the monoclonal antibodies described previously, the native collagen fragments must first be unwound and solubilized because in their helical state, the epitopes which can bind to the antibody are not accessible. Various techniques can be used to achieve this purpose. Among others is the proteolytic cleavage and solubilization of native collagen by selected enzymes. Among these enzymes, proteinase K is a preferred choice although when it is used, it is necessary to heat the mixture of proteinase K and the solubilized collagen to ensure complete unwinding of the helical chains as well as destroy the proteinase K activity.

The use of the enzymes referred to above is important as they permit the measurement of the degree of cartilage degradation in a given biological sample.

Assay method and kit

The purpose of the assay developed in the context of the present invention is three-fold. Firstly, it can be used to measure collagen degradation products in a biological sample through the use of the monoclonal antibodies described above. Secondly, it can be used to determine the amount of cartilage degradation in tissues by combining these antibodies with enzymes having the ability to cleave unwound collagen as well as means for unwinding remaining native helical collagen. Thirdly, it can be used to measure total collagen content in a biological sample.

In situations where it is desired to monitor collagen degradation in a biological sample, such as a body fluid, the monoclonal antibody is contacted with the sample and incubated for a period of time sufficient to provoke the desired immunological reaction. In most instances, the preferred product of cartilage breakdown that is monitored in body fluids is type II collagen, although it is to be appreciated that the degradation of other collagens such as type I collagen can also be monitored using such an approach.

The evaluation of collagen unwinding in a body fluid can be carried out through a kit which first comprises a monoclonal antibody having the characteristics described previously.

The kit also comprises a solid support for binding proteins. A wide variety of solid supports can be used. Nitrocellulose sheets or similar materials can be used but in particular, it is contemplated to use as the solid sorbent a plastic material such as polystyrene, a polyvinyl or other plastics which could include polyethylene, polypropylene, nylon and derivatized glass. The proteins that are bound to the solid support can be either the antibody itself, the synthetic peptide against which the antibodies were raised or heat-denatured collagen.

Also, the kit comprises a labelled antibody to measure the binding of the monoclonal antibody to unwound

collagen. This antibody can be radioactively or enzymatically labelled through techniques that are within the knowledge of the person skilled in the art. Preferred in the context of the present invention is an alkaline phosphatase-conjugated antibody used in combination with an alkaline phosphatase substrate which produces a coloured reaction product.

In situations where the determination of cartilage breakdown is conducted based on a tissue sample, it is not possible to simply react the tissue sample with a monoclonal antibody such as the one described above because there is a strong likelihood that denatured helical collagen domains might be retained in the tissue by cross-linking and fibrilar packaging. To address this problem, the biological sample is first contacted with an enzyme having the ability to selectively cleave unwound collagens without cleaving the antibody-reactive epitope. The fragments of unwound collagen are then extracted from the biological sample to produce an extract of unwound collagen fragments. This extract can then be assayed by being contacted with the monoclonal antibody described above. However, in order to accurately quantitate the degree of unwinding, the amount of wound collagen that is extracted by the above described enzyme (that selectively cleaves unwound collagen) should also be measured. This is done by treating an aliquot of the enzyme extract to solubilize and unwind any native collagen contained therein. This aliquot can then be assayed by being contacted with the monoclonal antibody described above.

Since the biological sample to be analyzed is a tissue sample, if one wishes to gain information on the level of cartilage degradation in this particular tissue, it is necessary to assess the total amount of collagen present in the sample. In order to do so, the biological sample is further treated to solubilize and unwind remaining native collagen that it contains. Native collagen can be solubilized and unwound by incubating the sample with an

enzyme having the ability to solubilize helical collagen from cartilage without cleaving the antibody reactive epitope and by submitting the enzyme and solubilized collagen to heat denaturation. The biological sample is then assayed using the monoclonal antibody described above. It is then possible to evaluate the degree of unwinding of collagen by comparing the immunological reaction of the monoclonal antibody with the unwound collagen fragments extracted, to the reaction with the remaining native helical collagen which has been solubilized and unwound.

Thus, when the kit of the present invention is to be used for quantitative measurement of cartilage degradation from a tissue sample, it further comprises an enzyme having the ability to selectively cleave unwound collagen chains in the biological sample without cleaving antibody-reactive epitope on the collagen chains. The kit can further comprise another enzyme having the ability to solubilize helical collagen from cartilage without cleaving antibody-reactive epitope. It is to be noted that although the enzymes referred to above are not absolutely necessary to carry out the method of the present invention when a biological fluid is assayed, these enzymes could be optionally used in body fluid samples.

When it is desired to measure total collagen present in a biological sample, it is first necessary to treat the biological sample to solubilize and unwind all collagen present. As mentioned previously, heat denaturation and certain proteases can be used to unwind collagen present in the sample in its native form. The use of an enzyme having the ability to selectively cleave unwound collagen becomes unnecessary since the problem relating to cross-linking and fibrillar packaging is not encountered, as all collagen present is immediately unwound. Once all collagen has been unwound and solubilized, it can be assayed by being contacted with the monoclonal antibody described above.

If it is to be used in total collagen degradation, the kit of the present invention comprises the monoclonal antibody having the characteristics described previously, a solid support for binding proteins as well as a labelled antibody to measure the binding of the monoclonal antibody to unwound collagen. Ideally, the kit also comprises means for unwinding the collagen present in the biological sample to be analyzed.

In vivo detection of collagen damage in cartilage

The monoclonal antibody developed in the context of the present invention can also be used for detecting in vivo the presence of damaged collagen in joints. By employing the Fab portion of the antibody, which can penetrate cartilage, *in vivo* diagnostic imaging of collagen degradation in joints can be performed. For this application, the Fab portion of the antibody is preferably radiolabeled with a short lived radioactive γ emiter such as Yttrium or Indium. The labelled antibody is injected in patients and radiological imaging with a γ camera allows the detection of collagen damage in cartilage in joints.

The following examples are provided to further illustrate the present invention.

EXAMPLE 1

PREPARATION OF IMMUNOGENIC PEPTIDE CB11B.

A. IDENTIFICATION OF AN IMMUNOGENIC EPITOPE ON PEPTIDE CB11 AND PREPARATION OF A SYNTHETIC PEPTIDE

Five hydrophilic domains (hydrophilicity less than -1.0) were identified from the hydrophobicity/hydrophilicity profile of the CB11 peptide of human type II collagen. The sequence of type II collagen is shown in Figure 1 and SEQ ID NO:1. The CB11 peptide (referred to as CNBr:11 on Figure 1) is shown to span from aa 255 to aa 533 and is shown in SEQ ID NO:2. The hydrophobicity/hydrophilicity plot for the CB11 peptide is shown in Figure 2. A 21 amino acid sequence from one of these domains satisfied the three additional criteria for peptide sequence selection, that is no hydroxylysine residues, conservation of the

peptide sequence between species and minimal homology with sequences from other α-chains of other collagens besides type II with any other known protein sequence. The sequence of this peptide, termed CB11B, is shown in Figure 3 and SEQ ID NO:3. The peptide was synthesized with an additional N-terminal cysteine for conjugation to ovalbumin and a C-terminal tyrosine (for possible radiolabelling with ^{125}I) at a 0.25 mmole scale, using standard FAST-MOC chemistry on an Applied Biosystems Model 431A solid phase peptide synthesizer. Crude peptides were purified by reverse phase chromatography (Prep-10 aquapore C8 column, Applied Biosystems) using an acetonitrile gradient in 0.1% trifluoroacetic acid.

B. COUPLING OF SYNTHETIC PEPTIDE TO OVALBUMIN

The bifunctional reagent bromoacetic acid-N-hydroxy succinimide ester (Sigma) was prepared fresh at 60 µg/ml in dimethyl formamide. For coupling, 0.2 ml of this solution was added, dropwise, with continuous stirring to 2 ml of ovalbumin (25 mg/ml; Sigma) dissolved in 0.1M phosphate buffer, pH 7.0, containing 1 mM EDTA, at 4°C. The mixture was then allowed to equilibrate to room temperature over 30 minutes. The activated ovalbumin was separated from unreacted coupling reagent by gel filtration using a Sephadex G25 column (27 X 2.2 cm) eluted with the EDTA/phosphate buffer described above. The peptide to be coupled was dissolved in 0.1M phosphate buffer, pH 7.0 and 5 mg was added to approximately 4 mg of activated ovalbumin (about 1.5 ml of the Sephadex G25-eluate peak fractions). The mixture was stirred for 2 h at room temperature and then incubated overnight at 4°C. The conjugate solution was dialysed exhaustively against azide-free phosphate buffered saline (PBS). Success of the coupling reaction was determined by confirming a reduced electrophoretic mobility of the peptide-ovalbumin conjugate compared to unconjugated ovalbumin.

EXAMPLE 2

PREPARATION OF MONOCLONAL ANTIBODIES TO SYNTHETIC PEPTIDE CB11B.

A. PREPARATION OF HYBRIDOMA CELLS

Four 8-10 week-old female BALB/c mice were each immunized by intra-peritoneal (i.p.) injection with 100 µg of the synthetic peptide of Example 1 conjugated to ovalbumin in 100 µl PBS and emulsified with 100 µl of Freund's complete adjuvant. Each mouse received four subsequent i.p. immunizations with the same quantity of antigen emulsified in Freund's incomplete adjuvant, at two week intervals. Serum samples were collected, 10 days after the second and fifth immunizations, by retro-orbital bleeding and tested in an ELISA for positive reactivity with both the synthetic peptide of Example 1 and heat denatured type II collagen (HDC) and negative reactivity against helical (native) type II collagen. The mouse showing the best serum antibody response was given a sixth immunization, with unconjugated peptide in azide-free PBS (100 µg i.p. and 100 µg intravenous). Three days later, the animal was sacrificed by asphyxiation and the spleen removed. The splenocytes were isolated by gently grinding the tissue through a sterile metal mesh. They were fused to SP2/0 myeloma cells and cloned as described by de Fazekas and Scheidegger (4) hereby incorporated by reference. Hybridoma cells from the best clone (Col 2-3/4m) were injected i.p. into female retired breeder BALB/c mice that had been primed with pristane (Sigma). Ascitic fluid containing the monoclonal antibody COL2-3/4m which is reactive with both CB11B synthetic peptide of Example 1 and HDC was harvested after 8-10 days. The antibody isotype was determined using a commercial isotype screening kit (Southern Biotechnology Inc., Birmingham, AL).

The hybridoma was deposited under the Budapest Treaty at the American Type Culture Collection (ATCC) on November 17, 1992 and was assigned ATCC designation HB 11202.

B. CHARACTERISTICS OF MONOCLONAL ANTIBODY COL2-3/4M

COL2-3/4m was found to have an IgG_1 isotype. The specific epitope sequence recognized by the monoclonal was identified by synthesizing short, overlapping peptides from within the sequence of CB11B and testing these for reactivity with the monoclonal (data not shown). The epitope was identified as peptide CB11B/H, a 13 amino acid sequence containing three hydroxyproline residues (Figure 3b and SEQ ID NO:4). Shortening of the sequence at the amino-terminus by removing one amino acid reduced reactivity with COL2-3/4m by 70% and shortening at the carboxy terminus reduced reactivity by 33% when one amino acid was removed and 92% when two were removed (data not shown). Peptide CB11B/H retained full reactivity with antibody COL2-3/4m in an inhibition assay, even when proline was substituted for any one of the hydroxyproline residues (data not shown). The sequence of epitope CB11B/H was compared to those of all proteins included in release 22 of the Swiss-Prot protein sequence database, using MacMolly Tetra Software. (Soft Gene GmbH, Berlin, Germany). The only protein sequences found to contain the epitope CB11B/H were those of the α_1(II) and the α_3(XI) chains.

EXAMPLE 3

PURIFICATION OF COLLAGENS

Bovine and human type II collagen and bovine types I and III collagens were prepared by differential salt precipitation, as described by Dodge and Poole (1) and Epstein (5). Bovine type XI collagen was prepared following the technique described by Eyre D.R., et al. (6). Type X collagen can be purified by the method described by Kirsch T. et al. (7).

EXAMPLE 4

PROTEOLYTIC CLEAVAGE OF NATIVE TYPE II COLLAGEN BY COLLAGENASE.

Bovine native type II collagen was initially dissolved in 0.5 M acetic acid and the diluted to a final concentration of 0.5 mg/ml in 100 mM Tris-HCl, pH 7.6,

containing 10 mM $CaCl_2$ and recombinant human collagenase which had been activated by incubation with 0.25 mM aminophenyl mercuriacetate for 10 minutes at 37°C. The final molar ratio of collagenase to collagen was 1:5 and the control tube contained type II collagen in the APMA-buffer with no collagenase. The samples were incubated for 20 hours at 30°C and the collagenase was then inhibited by the addition of EDTA to each tube to a final concentration of 10 mM.

EXAMPLE 5

ELECTROPHORESIS AND IMMUNOBLOTTING OF PURIFIED COLLAGENS.

SDS-PAGE of purified collagen was performed using a 7.5%, 1 mm thick, 7 cm x 8 cm mini-Protean gel, as described by Dodge and Poole in 1989 J. Clin. Invest. 83:647-661. The electrophoresed samples were transferred to a nitrocellulose membrane which was then blocked overnight at 4°C with PBS containing 3% w/v serum albumin (BSA) (PBS-3% BSA). The membrane was then incubated for 1 hour at room temperature with the COL2-3/4m monoclonal antibody of Example 2 or normal mouse ascitic fluid, each diluted 1 in 100 with PBS-3% BSA. After 3 washes with PBS-0.1% Tween-20, the membrane was incubated for 30 minutes at room temperature with the alkaline phosphatase conjugated goat anti-mouse second-step antibody described above, diluted 1 in 100 with PBS-3% BSA. The membrane was washed well with PBS-Tween and once with distilled water. Alkaline phosphatase substrate solution prepared from a commercial kit (Bio-Rad) was added and incubated with the membrane at room temperature until optimal colour had developed. Further reaction was stopped by washing off the substrate solution with distilled water.

Western blotting analysis confirmed that COL2-3/4m reacts with denatured type II collagen but not with denatured collagen types I, III or X (Figure 4). It did cross-react with the α3 chain of type XI collagen, but not with the α1 (XI) or α2 (XI) chains (Figure 4). In a separate immunoblot analysis, the monoclonal antibody was

shown to react with the 3/4 piece product of collagenase-cleaved type II collagen, but not the 1/4 piece product (Figure 5). This confirms that the reactive epitope is on the 3/4 piece product of collagenase-cleaved type II collagen.

EXAMPLE 6

ELISA ASSAY FOR MOUSE ANTIBODIES TO SYNTHETIC PEPTIDE CB11B AND HDC.

HDC was prepared by heating a 1 mg/ml solution of type II collagen for 20 minutes at 80°C. The wells of Immulon-2 ELISA plates (Dynatech) were each coated with 2 µg of synthetic peptide CB11B or HDC, in 50 µl of 0.1 M carbonate buffer (pH 9.2), by passive adsorption for 24-48 h at 4°C. The plates were washed three times with PBS containing 0.1% v/v Tween-20 (PBS-Tween) and unreacted sites were blocked by incubation with 50 µl/well of 1% w/v bovine serum albumin (PBS/BSA) for 30 minutes at room temperature. The plates were washed once with PBS-Tween and 50 µl of diluted mouse serum or undiluted hybridoma culture supernatant was added to individual wells. After incubation for 90 minutes at 37°C the plates were washed three times with PBS-Tween and then alkaline-phosphatase-conjugated goat anti-mouse IgG, IgM and IgA (Zymed) prepared at a 1:500 dilution in PBS-BSA-Tween was added at 50 µg/well. The plates were incubated for 90 minutes at 37°C and then washed three times with PBS-Tween and once with distilled water. Alkaline phosphatase substrate (Sigma) was prepared fresh at 0.5 mg/ml in 9.6% v/v diethanolamine, 49 µg/ml $MgCl_2$, pH 9.8 and incubated in each well for 20-30 minutes at 37°C. The absorbance was measured at 405 nm on a Multiskan plus MKII plate reader (ICN/Flow).

EXAMPLE 7

INHIBITION ELISA FOR DENATURED TYPE II COLLAGEN.

A. ASSAY PROCEDURE

Heat denatured collagen (HDC) or standard peptide CB11B were preincubated with antibody in 96-well round

bottom Linbro microtiter plates (Titertek) which were first coated with 100 µl/well of PBS-BSA, as described above. The outermost wells were not used, to minimize the effects of evaporation. There were six non-specific binding (NSB) wells on each plate, which contained 100 µl each of Tris buffer. Fifty µl per well of monoclonal ascitic fluid, diluted appropriately (1 in 5,000 - 1 in 10,000) with 50 mM Tris, pH 7.6 (Tris buffer) to provide a detectable but inhibitable level of binding was added to 54 wells of the pre-incubation plates. The antibody in 6 of the 54 wells on each plate was mixed with 50 µl/well of Tris buffer, to indicate maximum binding in the absence of inhibitory epitope (MB wells). The antibody in all 48 test wells was mixed with 50 µl/well of standard CB11B peptide or samples containing denatured collagen, diluted appropriately with Tris buffer. All standards and samples were tested in duplicate wells. The plates were sealed with parafilm and incubated overnight at 37°C in a humidified incubator. A multichannel pipette was used to transfer 50 µl of each pre-incubated sample to the equivalent well of an Immulon-2 ELISA plate, coated with 2 µg/well HDC and blocked with PBS-BSA as described above. It was essential to transfer all the samples from any one plate within a short space of time (about 45 seconds) in order to maximize accuracy. The ELISA plates were each incubated for exactly 30 minutes at room temperature and then washed three times with PBS-Tween. Second antibody and alkaline phosphatase substrate were prepared and added as described above, except that the plates were incubated with the second antibody for 2 hours. The mean of absorbance from the 6 NSB wells was substracted from the absorbance value of all other wells on the sample plate. The percentage inhibition of binding by samples or standards was calculated relative to the mean absorbance from the 6 MB wells on the same plate, which represented 0% inhibition (100% binding).

B. CHARACTERISTICS OF THE CB11B INHIBITION ELISA

One sample of HDC was assayed for CB11B 15 times on each of 10 ELISA plates. From this data, the mean coefficient of variation was calculated as 9% for within-plate analysis and 10% for between-plate analysis.

A typical standard curve, plotted as log % inhibition against log CB11B concentration, is shown in Figure 6. Samples of denatured type II collagen were diluted such that the level of inhibition in the assay fell within the range 20-70%. This represents 0.35-3.5 µg/ml CB11B (approximately 17.5-175µg/ml denatured type II collagen) in the diluted sample.

Figure 7 shows the concentration/response profiles for inhibition in the assay by HDC compared with CB11B. On a µg/ml basis the profiles were parallel (Figure 7a) and on a molar basis the range of concentrations of antigen producing inhibition were found to be similar for CB11B and HDC (Figure 7b). Therefore the concentration of CB11B can be used to estimate the concentration of denatured type II collagen, or fragments thereof containing this epitope.

The traditional method of estimating collagen concentrations is on the basis of hydroxyproline content. Therefore, a comparison was made of the concentrations of CB11B and hydroxyproline in different HDC samples. The results (Figure 8) show a very good correlation between the two methods ($r = 0.998$; $p < 0.0001$).

It was important to demonstrate that the inhibition ELISA did not recognize intact, native type II collagen. The data in Figure 9 show that when preparations of bovine type II collagen were tested at various concentrations in the native or heat-denatured forms, only the denatured collagen could be detected.

Heat denatured type I and type III collagen were also tested by the above described inhibition ELISA method. Table I provided below shows that heat denatured type I and type III collagens were not detectable in the assay,

confirming the finding from Western blotting that COL2-3/4m does not react with type I or type III collagens.

TABLE I

[HDC] (ug/ml)	[CB11B] BY INHIBITION ASSAY (ug/ml)		
	TYPE I HDC	TYPE II HDC	TYPE III HDC
31.25	0	0	0
62.5	0	1.53	0
125	0	2.19	0
250	0	5.34	0

EXAMPLE 8

DETECTION OF TYPE II COLLAGEN IN HUMAN SERA AND SYNOVIAL FLUIDS.

An assay procedure similar to the assay outlined in Example 7 was used to evaluate the presence of collagen II CB11B epitope in sera and synovial fluids (SF) of normal children, children with chondrodysplasias, normal adults and patients with osteoporosis, osteoarthritis and rheumatoid arthritis.

The relative concentrations of the CB11B in sera of normal children (1-12 years) and adults (40-90 years) and in children with chondrodysplasias is shown in Figure 10. This data indicates relatively high levels of type II cartilage degradation in most children, significantly reduced levels in some adults (39%) but elevated 'childhood' levels in other 'healthy' adults (61%). Hence, the adult levels fall into two categories, one of which is similar to the levels observed in most normal children. In contrast, levels in children with chondrodysplasias, who ordinarily exhibit impaired growth, are generally lower than those found in normal children.

In Figure 11, relative concentrations of the CB11B epitope in sera from normal adults and patients with osteoporosis (OP), rheumatoid arthritis (RA) and osteoarthritis (OA) are shown. Levels in OP and OA are mostly

comparable to those observed in the 'elevated' normal group. In RA a wide variation in levels was observed. It is possible that the 'elevated' normal adult group may exhibit increased cartilage collagen degradation which predisposes to degenerative changes observed in OA. The data also show that there is a significant increase in cartilage collagen degradation in OP.

Figure 12 shows the relative concentrations of the CB11B epitope in synovial fluid (SF) and serum of individual patients with rheumatoid arthritis (RA). The levels are always higher in SF. In Figure 13, relative concentrations of the CB11B epitope in different patients and normal groups in sera and synovial fluids (SF) are shown. The elevations in RA and OA SF over serum levels can be seen.

In order to validate the serum and synovial fluid assay experiments described above, normal human serum (NHS) and sera from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) were diluted and assayed for the presence of the collagen II CB11B epitope. Results shown in Figure 14 demonstrate that dilution of NHS and sera from patients with RA and OA produces inhibition profiles which exhibit parallelity with inhibition curves for CB11B in the absence of serum. This data demonstrates that the assay of the present invention can be used to quantitate levels of CB11B in sera.

Furthermore, 'spiking' experiments were performed whereby a known amount of denatured collagen was added to normal human sera and rheumatoid sera, following which recovery was measured. Recovery is defined as the sum of collagen CB11B present in the serum and collagen CB11B added. Percentage recoveries (means $\pm$ standard deviations of n determinations) were as follows:

RA synovial fluid:	89 ± 20	(n = 5)
RA serum:	94 ± 16	(n = 5)
normal serum:	85 ± 4	(n = 4)

EXAMPLES 9 - 12 - TESTING OF BIOLOGICAL TISSUE

Collagen degradation was also measured in various biological tissues including bovine cartilage discs, bovine cartilage explant cultures and human cartilage. Therefore, a method was developed to extract denatured type II collagen from the tissue in order to use it in the inhibition ELISA assay described in Example 7. The enzyme chymotrypsin was shown to extract unwound collagen from a cartilage sample without degrading the epitope recognized by monoclonal antibody COL2-3/4m. In order to quantitate the amount of unwinding or degradation of collagen in a biological sample, it is necessary to determine the amount of wound collagen in the sample. Therefore, once the unwound collagen was extracted from the samples, the samples were treated with proteinase K to solubilize and unwind remaining native collagen which could also be measured using the inhibition ELISA described in Example 7.

Before using the enzymes chymotrypsin and proteinase K on biological tissues, it was first determined that these enzymes did not cleave the epitope recognized by the monoclonal antibody COL 2-3/4m.

EXAMPLE 9

A. PROTEOLYTIC CLEAVAGE OF HDC IN A BIOLOGICAL SAMPLE BY CHYMOTRYPSIN AND PROTEINASE K.

Aliquots of HDC at a concentration of 0.25 mg/ml were prepared in Tris buffer containing 0.5 mg/ml of α-chymotrypsin or proteinase K (both from Sigma). Control tubes contained only 0.25 mg/ml HDC, or 0.5 mg/ml chymotrypsin or 0.5 mg/ml proteinase K. The samples were all incubated overnight at 37°C and then boiled for 10 minutes to inactivate the proteases. Degradation of the HDC by each protease was confirmed by SDS-PAGE. The degraded collagens and control samples were each tested for reactivity in the inhibition ELISA for denatured type II collagen.

B. PROTEOLYTIC CLEAVAGE OF HDC AND CARTILAGE

As shown in Table II below, digestion of HDC with either chymotrypsin or proteinase K had no effect on its reactivity in the inhibition ELISA.

TABLE II

COMPETING ANTIGEN	[CB11B] by inhibition assay (µg/ml)
250 µg/ml HDC	5.0
250 µg/ml HDC + 500 µg/ml CHYMOTRYPSIN	5.7
250 µg/ml HDC + 500 µg/ml PROTEINASE K	6.0

This indicates that the CB11B epitope is not cleaved by these proteases. The inhibited proteases themselves did not have any inhibitory activity in the assay. Since chymotrypsin, like all mammalian proteases apart from collagenase, cannot cleave intact, triple helical collagen, as confirmed by the data in Table III this enzyme can be used to selectively degrade and solubilize any collagen in cartilage which has already been unwound *in situ*. Conversely, cartilage can be fully solubilized by incubation with proteinase K at 56°C (its optimal temperature) and the extracted collagens can then be completely unwound by heat denaturation.

Table III

[COLLAGEN] (ug/ml)	[CB11B] BY INHIBITION ASSAY (ug/ml)			
	HDC	HDC + 500ug/ml CHYMOTRYPSIN	NC	NC + 500ug/ml CHYMOTRYPSIN
62.5	1.27	1.55	0.2	0
125	2.1	2.2	0	0
250	5.8	6.6	0.1	0

EXAMPLE 10

ASSAY OF CARTILAGE DISCS FOR DENATURED COLLAGEN.

A. PREPARATION OF CARTILAGE DISCS

Bovine foetal epiphyseal cartilage was obtained from the proximal tibiae of fetuses, determined to be 200-250 days old as described by Pal S. et al. (8), shortly after the sacrifice of pregnant cows at a local slaughter house. A stainless steel cork borer was used to make full-depth plugs of cartilage with a diameter of 6 mm. The plugs were placed in a custom-made tissue slicer, designed to make reproducible discs of tissue, each 1 mm thick, using a razor blade. Discs were cut from the growth plate fracture face of the physis with the metaphysis through to the articular surface (a total depth of 7 mm) and the relative position of each one was noted. A single disc of 4.8 mm diameter was taken from each of the larger ones using the appropriate-sized borer. The resulting 1 mm x 4.8 mm discs were weighed prior to tissue culture or extraction with proteases: the approximate mean weight was 30 mg. Parallel plugs were used for the preparation of histological sections so that growth plate, epiphyseal cartilage and articular cartilage could each be accurately located.

B. EXTRACTION OF DENATURED COLLAGENS FROM CARTILAGE.

Freshly isolated or cultured discs of bovine foetal epiphyseal cartilage were distributed into Eppendorf tubes. To each tube was added 0.5 ml of 1 mg/ml α-chymotrypsin in Tris buffer containing 1 mM iodoacetamide, 1 mM

EDTA and 10 µg/ml pepstatin-A. The samples were incubated overnight at 37°C and the chymotrypsin was then inhibited by addition of 200 µl per tube of 0.4 mg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) in Tris buffer containing 4% v/v ethanol. The samples were centrifuged and the chymotrypsin extract was separated from the tissue residues. Each residue was then digested with 0.5 ml of 1 mg/ml proteinase K in Tris buffer containing the same protease inhibitors as described above. After digestion overnight at 56°C, no residue remained. The proteinase K was inactivated and the extracted collagen fully unwound by boiling the samples for 10 minutes. In order to measure any wound collagen extracted by chymotrypsin, 300 µl of each chymotrypsin extract was mixed with 100 µl of the 1 mg/ml proteinase K solution and incubated and then inhibited as described for cartilage residues. Extracts were stored at 4°C prior to assay for denatured collagen.

The samples prepared above were assayed for denatured collagen according to the assay described in Example 7. The results are shown in Figure 15. Figure 15 shows the percentage denatured (unwound) collagen in serial 1mm thick discs starting from the junction (fracture face) of the hypertrophic zone of the growth plate with the metaphysis (0-1mm) and extending through the epiphyseal cartilage into superficial articular cartilage (6-7mm). An elevation in the proportion of unwound type II collagen is observed in the hypertrophic zone, compared to other cartilages.

EXAMPLE 11

ASSAY OF CARTILAGE EXPLANT CULTURES FOR DENATURED COLLAGEN

A. PREPARATION OF CARTILAGE EXPLANT CULTURES

Cartilage discs were cultured in serum-free Dulbecco's modified Eagles medium (DMEM). The freshly isolated cartilage discs were individually washed 3 times with DMEM containing 5 µg/ml Fungizone (Flow laboratories) and once with regular DMEM. The discs were distributed into the wells of 48-well tissue culture plates (Costar). Discs cut from a defined distance from the growth plate fracture

face were used. Each well contained one disc in 500 µl DMEM. Ascorbate was added to all cultures at a final concentration of 50 µg/ml. Where indicated recombinant human IL-1ß (Upjohn) was added at 200 U/ml, together with 1 mg/ml of bovine serum albumin (BSA;Sigma). Control wells received only BSA and ascorbate. The explants were cultured for 6 days at 37°C in an atmosphere of 5% CO_2/air. Media were changed every 2 days with the addition of fresh IL-1ß as necessary. The media and cartilage discs were stored at -20°C prior to extraction or immunohistochemical analysis. Media samples or tissue extracts were assayed for denatured (unwound) and total type II collagen as described for the cartilage discs in Example 10.

EXAMPLE 12

DETECTION OF TYPE II COLLAGEN DEGRADATION IN HUMAN CARTILAGE.

The purpose of this study was to analyze type II collagen denaturation (unwinding) in whole depth samples resulting from *in vivo* cleavage in human femoral condylar articular cartilages. Normal and osteoarthritic (OA) persons were studied.

Cartilage discs, prepared as described in Example 10, were routinely extracted, first with chymotrypsin and then with proteinase K, as described previously in Example 10. Levels of denatured (unwound) type II collagen were estimated by measuring CB11B in the chymotrypsin extract. Extraction of intact (wound, native) type II collagen by chymotrypsin was determined by measuring CB11B in the proteinase K-digested chymotrypsin extract and substracting from this value the amount of CB11B extracted by chymotrypsin and immunoreactive in an unwound form. That the chymotrypsin extracts may contain some wound collagen is shown in Table IV. Here the chymotrypsin extract has also been treated with proteinase K to unwind any helical collagen. In human adult cartilages, this amount was low (2.8 - 3.3% of the total chymotrypsin extractable type II collagen) but in fetal bovine articular cartilage and

growth plate it could very between 7.1 and 24.3% of the total chymotrypsin extractable collagen. These results demonstrate the importance of treating the chymotrypsin extract with proteinase K to distinguish between unwound and wound collagens.

Total type II collagen was estimated by measuring CB11B in the proteinase K-digested residue (after initial chymotrypsin extraction) and adding to this value the amount of CB11B (wound and unwound) extracted by chymotrypsin. Results are shown in Figure 16 which represents the amount of denatured (unwound) collagen as a percentage of the total collagen present in femoral condylar cartilages where each bar represents one patient. Six specimens (A to F) were taken from normal patients and twelve specimens (A to L) were taken from osteoarthritic patients. There was no denaturation found in specimen B. An increase in collagen denaturation above the highest normal value was observed in 8 of 12 OA patients. The TPCK-inhibited chymotrypsin and boiled proteinase K were found to have no inhibitory effect themselves in the CB11B inhibition ELISA.

Table IV

TISSUE TYPE	NATIVE TYPE II COLLAGEN IN CHYMOTRYPSIN EXTRACT (% OF TOTAL CHYMOTRYPSIN-EXTRACTABLE TYPE II COLLAGEN)		
	NUMBER OF SAMPLES	MEAN %	RANGE
NORMAL ADULT HUMAN CARTILAGE	6	2.8	0-10.9
OSTEOARTHRITIC HUMAN CARTILAGE	23	3.3	0-34.6
BOVINE GROWTH-PLATE CARTILAGE	6	24.3	0-50
BOVINE FETAL ARTICULAR CARTILAGE	6	7.1	0-22.9

EXAMPLE 13

TYPE II COLLAGEN DEGRADATION IN OSTEOARTHRITIS

To study damage to type II collagen in articular cartilage in arthritis, we developed an immunoassay based on our earlier experiments described above. In this example, we describe and use this assay to provide the first quantitative evidence for increased damage to type II collagen in situ and decreased content of total type II collagen in OA cartilage.

MATERIALS AND METHODS

TISSUE.

Human articular cartilages were removed from the anterior (loaded) region of the femoral condyles of adult knee joints. OA cartilages were obtained at surgery from patients undergoing total joint arthroplasty. Site-matched nonarthritic articular cartilages of normal appearance were obtained at autopsy within 15 h postmortem from individuals with no known history, nor signs, of arthritic/joint abnormalities. Patient details and Mankin grades of the normal and OA cartilage specimens are shown in Table V. Previously, cartilage from these same sites have been analyzed for the proteoglycan aggrecan (9).

PREPARATION OF CARTILAGE PLUGS.

Since cutting cartilage can produce denaturation of type II collagen, care was taken to prepare defined plugs of cartilage using a specially made, standardized, stainless steel punch. This avoided variable results and the increased denaturation obtained by dicing the cartilage with a scalpel or by preparing frozen sections (data not shown). Wherever possible, a full depth slice of cartilage was taken from each femoral condyle (medial and lateral) and 2 full-depth cylindrical plugs, each approximately 4mm diameter x 2mm deep, were prepared from each slice using the steel punch. One of the plugs was used for the histological assessment of Mankin grade, as previously described (9,10). This did not include analysis of the calcified cartilage, hence the maximum grade in the group

of OA cartilages was only 10. The other plug was used for the extraction and assay of type II collagen. In some experiments the full depth plugs were approximately divided into an upper and a lower half using a scalpel. The upper 1mm included the articular surface and upper-mid zone whilst the lower 1mm consisted of lower-mid and deep zone cartilage. It was essential to standardize the tissue sample preparation in this way since cutting the cartilage causes denaturation of type II collagen.

PROTEOLYTIC CLEAVAGE OF NATIVE TYPE II COLLAGEN BY RECOMBINANT INTERSTITIAL COLLAGENASE.

Bovine native type II collagen was initially dissolved in 0.5M acetic acid and then diluted to a final concentration of 0.5mg/ml in 0.1M Tris-HCl, pH 7.6, containing 10mM $CaCl_2$ and recombinant human interstitial collagenase (MMP-1; kindly donated by Dr. Michael Lark, Merck, Sharpe and Dohme, Rahway NJ) which had been activated by incubation with 0.25mM aminophenyl mercuriacetate in the same buffer for 10 min at 37°C. The final molar ratio of human collagenase to collagen was 1:5. The control contained type II collagen in buffer with no collagenase. The samples were incubated for 20 h at 30°C then the collagenase was inhibited by the addition of 10mM EDTA (final concentration). The samples were tested for immunoreactivity by immunoblotting and immunoassay.

PROTEOLYTIC CLEAVAGE OF HDC AND α1(II)CB11B PEPTIDE BY CLOSTRIDIAL COLLAGENASE.

HDC and α1(II)CB11B were dissolved in Tris containing 5mM $CaCl_2$ and 14U/ml chromatographically purified collagenase form III (bacterial collagenase) from Clostridium histolyticum (Advance Biofactures Corp., Lynbrook, N.Y.). Controls contained HDC or α1(II)-CB11B in Tris with 5mM $CaCl_2$ but no collagenase, or collagenase in Tris with 5mM $CaCl_2$ but no HDC, nor α1(II)CB11B peptide. All the tubes were incubated overnight at 37°C and the collagenase was then inhibited by the addition of EDTA to a final concentration of 0.1M. The samples were each tested for

reactivity in the inhibition ELISA for denatured type II collagen.

PROTEOLYTIC CLEAVAGES OF HDC BY α-CHYMOTRYPSIN AND PROTEINASE K.

Aliquots of HDC at concentrations of 0.25mg/ml or less were prepared in Tris containing 0.5mg/ml of type VII α-chymotrypsin or proteinase K (both from Sigma). Control tubes contained only HDC, or 0.5mg/ml α-chymotrypsin or 0.5mg/ml proteinase K. The samples were all incubated overnight at 37°C (α-chymotrypsin) or 56°C (proteinase K). The α-chymotrypsin was inactivated by addition of 115µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma) in Tris, predissolved in ethanol (4% v/v, final concentration). Proteinase K was inactivated by boiling for 10 min. Degradation of the HDC by each proteinase was confirmed by SDS-PAGE (see below). The degraded collagens and control samples were each tested for reactivity in the inhibition ELISA for denatured type II collagen.

EXTRACTION AND ASSAY OF NATIVE AND DENATURED COLLAGEN FROM CARTILAGE PLUGS.

Since α-chymotrypsin, like all mammalian proteinases apart from interstitial collagenase, cannot cleave intact, triple helical type II collagen, this enzyme can be used to selectively degrade any collagen in cartilage which has already been denatured in situ. Conversely, cartilage can be fully solubilized by incubation with proteinase K at 56°C (its optimal temperature). Complete denaturation of the extracted collagen can then be ensured by heating. Therefore cartilage plugs were routinely extracted, first with α-chymotrypsin and then proteinase K. The plugs were distributed individually into Eppendorf tubes. To each tube was added 500µl of 1mg/ml α-chymotrypsin in Tris containing 1mM iodoacetamide, 1mM EDTA and 10µg/ml pepstatin-A (all from Sigma). The samples were incubated overnight at 37°C. The α-chymotrypsin was inhibited by addition of 200µl per tube of 0.4mg/ml TPCK in Tris containing 4% v/v ethanol. The α-chymotrypsin extract was separated from the

residue. Each residue was then digested with 500µl of 1mg/ml proteinase K in Tris containing the same protease inhibitors as described above. After digestion overnight at 56°C, no residue remained. The proteinase K was inactivated and the extracted collagen fully denatured by boiling the samples for 10min. In order to measure any native collagen extracted by α-chymotrypsin, a 300µl aliquot of each α-chymotrypsin extract was mixed with 100µl of the 1mg/ml proteinase K solution and incubated and then boiled as described for cartilage residues. The TPCK-inhibited α-chymotrypsin and boiled proteinase K had no inhibitory effect themselves in the inhibition ELISA.

DIGESTION OF EXTRACTED COLLAGEN PEPTIDES WITH CLOSTRIDIAL COLLAGENASE TO CONFIRM SUSCEPTIBILITY OF THE EXTRACTED CB11B EPITOPE TO CLEAVAGE BY THIS ENZYME.

Collagens were extracted from plugs of OA cartilage with α-chymotrypsin and proteinase K and the proteinases inhibited as described above. The extracts were each divided into two aliquots. To one of these was added 5mM$CaCl_2$ and 14U/ml clostridial collagenase and to the other, 5mM$CaCl_2$ only. A control tube contained collagenase in Tris with 5mM$CaCl_2$ but no cartilage extract. All tubes were incubated overnight at 37°C and the collagenase activity was inhibited with EDTA as described above. The samples were all tested for reactivity in the inhibition ELISA for denatured type II collagen.

EXTRACTION OF CARTILAGE PLUGS WITH GUANIDINIUM CHLORIDE TO DETERMINE THE CONTENT OF NON CROSS-LINKED COLLAGEN.

In one experiment, two adjacent full-depth plugs were taken from each of 5 OA cartilages. One of the plugs from each specimen was extracted with α-chymotrypsin as described above. The other plug from each specimen was extracted for 72h at 4°C with gentle rocking in 4mM guanidinium chloride, 0.1M Tris-HCl, pH7.3 containing 1mM iodoacetamide, 1mM EDTA and 10µg/ml pepstatin-A. The guanidinium chloride extracts were dialysed exhaustively against Tris using membrane with a molecular weight cut-off of

3,500. A 300µl aliquot of each of the α-chymotrypsin and guanidinium chloride extract was mixed with 100µl of 1mg/ml proteinase K, incubated overnight at 56°C and then boiled to inhibit the proteinase. All the extracts were tested for reactivity in the inhibition ELISA for denatured type II collagen.

ELECTROPHORESIS AND IMMUNOBLOTTING

SDS-PAGE of purified collagens was performed using 7.5% and 10%, 1mm thick, 7cm X 8cm mini-Protean gels, stained with either Coomassie brilliant blue or silver stain (Bio-Rad Laboratories, Mississauga, ON), as described by Dodge and Poole (1). The electrophoresed samples were transferred to a nitrocellulose membrane which was then blocked overnight at 4°C with PBS containing 3% w/v BSA (PBS-3% BSA) (1). The membrane was incubated for 1 h at room temperature with the monoclonal antibody or control mouse ascitic fluid diluted in PBS-3% BSA. After 3 washes with PBS-Tween, the membrane was incubated for 30 min at room temperature with the alkaline phosphatase conjugated goat anti-mouse second-step antibody described above, diluted 1 in 100 with PBS-3% BSA. The membrane was washed well with PBS-Tween and once with distilled water. Alkaline phosphatase substrate solution was prepared from a commercial kit (Bio-Rad) employing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. It was added and incubated with the membrane at room temperature until optimal colour had developed. Further reaction was stopped by washing off the substrate solution with distilled water.

DETERMINATION OF TYPE II COLLAGEN CONTENT BASED ON EPITOPE ANALYSIS.

The molecular weights of the human type II collagen α1-chain and of peptide α1(II)-CB11B were each calculated from their amino acid sequences using version 1.00 of Prosis software (Hitachi Software Engineering Co. Ltd., USA). It was assumed that there are 99 hydroxy-proline residues and 20 hydroxylysine residues for every 1000 total residues of the α1(II) chain (21). On this

basis, the molecular weights of the α1(II) chain and α1(II)-CB11B peptide were calculated as 98,291 and 2,231 respectively. Therefore, the μg/mg concentration of type II collagen extracted from cartilage was calculated by multiplying the concentration of extracted α1(II)-CB11B by a factor of 44.

STATISTICAL ANALYSIS.

Significance of differences in total and denatured collagen was tested using the Mann-Whitney U-test for comparison between groups and the paired t-test for comparison of upper 1mm with lower 1mm zones prepared from the same cartilage plugs.

DENATURATION OF TYPE II COLLAGEN AFTER CLEAVAGE BY INTERSTITIAL COLLAGENASE.

Cleavage of purified native type II collagen by recombinant human interstitial collagenase produced the characteristic 3/4 and 1/4 products, seen by SDS-PAGE, but only the 3/4 product was detected by immunoblotting with COL2-3/4m (Fig. 17A). When the collagenase-cleaved collagen was assayed for α1(II)-CB11B, it was found that 56% of the collagen had been denatured compared to just 11% denaturation in the native collagen control which had been incubated under the same conditions but without collagenase. The partial denaturation of the control collagen was due to the prolonged incubation (20h) at 30°C. There was no evidence to indicate that the α1(II)-CB11B epitope is cleaved by interstitial collagenase.

LOCATION OF α1(II)-CB11B ON CYANOGEN BROMIDE PEPTIDE CB11.

Since the α1(II)-CB11B epitope amino acid sequence only occurs in the CB11 peptide of type II collagen, the antibody should only recognize CB11. Cleavage of type II collagen with cyanogen bromide produced a number of peptides, including CB8, CB10 and CB11, as seen by SDS-PAGE and silver staining (Fig. 17B). The only peptide to be detected by immunoblotting with monoclonal COL2-3/4m was, as expected, based on the location of the epitope, CB11 (Fig. 17B).

EPITOPE IN PEPTIDE α1(II)-CB11B IS CLEAVED BY CLOSTRIDIAL COLLAGENASE.

When clostridial collagenase alone was inhibited with 100mM EDTA and assayed for α1(II)-CB11B it produced a small amount of inhibition, equivalent to a background level of 0.85µg/ml α1(II)-CB11B (Table VI). This inhibition was probably due to cleavage of some of the HDC bound to the ELISA plate, by residual clostridial collagenase activity. HDC or α1(II)-CB11B alone incubated in Tris with $CaCl_2$ at 37°C for 24h produced good inhibition in the assay for α1(II)-CB11B, but the amount of α1(II)-CB11B detected was reduced to the background level when HDC or α1(II)-CB11B were treated with clostridial collagenase (Table VI), demonstrating hydrolysis of the epitope by clostridial collagenase. Therefore this property can be used to confirm the identity of the epitope in ELISA inhibition assays of samples containing it.

PROTEOLYTIC CLEAVAGE OF HDC.

HDC was extensively cleaved into small, undetectable peptides by both α-chymotrypsin and proteinase K, as judged by SDS-PAGE (data not shown) but without loss of the epitope (Table II). The inhibited enzymes themselves did not have any inhibitory activity in the assay. These results again demonstrate a small increase in immunoreactivity on cleavage of the HDC.

TOTAL TYPE II COLLAGEN CONTENTS AND CONTENTS OF DENATURE TYPE II COLLAGEN IN FULL-DEPTH SAMPLES OF HUMAN FEMORAL CONDYLAR CARTILAGES.

The mean type II collagen concentration for the normal cartilages was 139.5µg/mg. Thus for normal cartilage the total type II collagen accounts for a mean (range) of 14.0% (9.2-20.8%) of the wet weight of the tissue. For OA cartilage the equivalent mean (range) value was 10.3% (7.4-15.0%). The total amount of type II collagen/mg wet weight was significantly decreased in OA compared to normal cartilage (Fig. 18).

The proportion of denatured type II collagen was significantly increased in OA compared to normal cartilage (Fig. 18). The mean values for % denatured collagen in normal and OA cartilages were 1.1% and 6.0% respectively.

There were no correlations between either total or % denatured type II collagen and the degree of cartilage degradation recorded as the Mankin grade of OA cartilages (Fig. 19).

DIGESTION OF EXTRACTED COLLAGEN WITH CLOSTRIDIAL COLLAGENASE.

It was important to demonstrate that inhibition observed in the α1(II)-CB11B ELISA by α-chymotrypsin and proteinase K extracts of human cartilage was due entirely to the α1(II)-CB11B epitope. Since the epitope is destroyed by treatment with clostridial collagenase (see above and Table VI), some extracts were treated with this enzyme as a control for specificity. There was a loss of immunoreactivity in each case (Table VII), demonstrating the specificity of the assay for the α1(II)-CB11B epitope in the cartilage extracts.

VARIATION IN TOTAL AND DENATURED TYPE II COLLAGEN WITH DEPTH OF CARTILAGE.

From cartilage in which specimens could be obtained (minimum thickness, 2mm), plugs were divided into the upper 1mm (articular zone) and lower 1mm (deeper zone; these are approximate thicknesses) using a scalpel and a specially constructed slicing bed. The total type II collagen concentration was higher in the deeper (lower 1mm) compared to more superficial (upper 1mm) cartilage for 4 of 5 normal and 5 of 8 OA specimens (Figs. 20A and 20C). The % denatured collagen did not vary significantly with depth in normal cartilage (Fig. 20B). In one sample there was a high level of denatured collagen in the more superficial cartilage and increased denaturation in the deeper cartilage too. In 2 of the 4 other specimens, denaturation was enhanced in the more superficial cartilage, otherwise it was similar in each zone or higher in the deeper cartilage.

For OA cartilage the % denatured collagen was significantly higher in the upper 1mm zone (Fig. 20D). This difference was seen in 6 of 8 specimens. Of the other 2 specimens one showed the opposite trend and the other exhibited similar denaturation in the upper and lower levels. There was no correlation between total or % denatured collagen in either the upper or lower zones and Mankin grade of the full-depth cartilage for normal and OA specimens (data not shown). The mean values of total and denatured collagen in the upper and lower 1mm zones are shown in Table VIII, for comparison of each zone in OA with the same zone in normal cartilage. In OA, total collagen content was only significantly reduced in the deeper cartilage of OA compared to normal. Similarly, type II collagen denaturation was only significantly increased in the lower zone of OA compared to normal cartilage. The differences in % denatured collagen in either region were not as marked as the changes seen in full-depth plugs. However it should be noted that for the depth-study, cartilage was taken from the thickest region of OA tissue and this may have been less degraded than cartilage from the thinner tissue sites.

GUANIDINIUM CHLORIDE EXTRACTS OF OA CARTILAGE.

In order to determine if the type II collagen extracted from cartilage by α-chymotrypsin was mostly derived from fibril-associated, cross-linked collagen or from non-fibrilar, non-cross-linked α-chains, a comparison was made of extraction by α-chymotrypsin and 4M guanidinium chloride. The latter is a chaotropic reagent which can only extract the non-fibrilar, non-cross-linked collagen molecules. Adjacent plugs of cartilage from 5 OA patients were extracted with α-chymotrypsin or guanidinium chloride. Far less type II collagen was extracted with 4M guanidinium chloride than with α-chymotrypsin. For the 5 OA specimens examined the mean $\pm$ SD total (native + denatured) type II collagen extracted by guanidinium chloride and α-chymotrypsin was 0.019 $\pm$ 0.003 and 0.164 $\pm$ 0.012 μg/mg wet weight respectively. Therefore the amount of type II collagen

extracted by 4M guanidinium chloride is 11.6% of that extracted by α-chymotrypsin, suggesting that most of the denatured material extracted with α-chymotrypsin is derived from cross-linked fibrils rather than a pool of newly synthesized, non-cross-linked α-chains or peptides thereof. Of the type II collagen extracted with guanidinium chloride or α-chymotrypsin, only a small proportion (10.5% and 7.3% respectively) was native collagen (detected after digestion with proteinase K). However, we have found that incubation of purified native type II collagen for 72h at 4°C in 4M guanidinium chloride, followed by dialysis into Tris, causes over 90% of the collagen to denature, as judged by assaying for α1(II)-CB11B (data not shown). Therefore, it is likely that most of the α1(II)-CB11B extracted with guanidinium chloride is contained within newly synthesized molecules that are denatured by the extraction procedure.

DISCUSSION

The degeneration of cartilage, which is an integral feature of both OA and RA, involves localized loss of the proteoglycan aggrecan, its degradation (9,11) and eventual loss of function of this tissue (12-14). The tissue is composed of a fibrillar organization of type II collagen (15) which endows cartilage with its tensile strength (16,17). With increasing age there is a progressive reduction of the tensile properties of femoral head cartilage (18). This is not observed in the talus of the ankle joint where the incidence of osteoarthritis is relatively low compared to that observed in the hip or knee (18). In the present study we show that damage to type II collagen, measured as denaturation (unwinding) of the triple helix, is detectable in adult human femoral condylar cartilage and is increased in OA cartilage. This damage in OA is accompanied by a net reduction in the total type II collagen content. Previously, a loss of tensile properties was observed in OA cartilage, indicative of damage to type II collagen (12-14). The present studies reveal that the reduced tensile strength in part relates to damage to

the triple helix of type II collagen leading to denaturation (unwinding). It also correlates with a net loss of this molecule in OA, previously indicated by a reduction in total cartilage collagen content measured as hydroxyproline (19). In contrast, this loss of collagen is not observed in RA femoral condylar cartilage, although there is a similar increase in type II collagen denaturation to that observed in OA (A. Hollander, T. Heathfield, I. Pidoux, W. Fisher, E. Bogoch and A.R. Poole, manuscript in preparation).

We also show that the damage to type II collagen of the femoral condylar cartilage in OA is most pronounced in the superficial and upper-mid zone (upper 1mm slice); this is where the proteoglycan aggrecan is first lost from the matrix in OA (15). Such a difference between upper and lower zones is not observed in normal cartilage. If we compare damage to the lower zone in normal and OA cartilages we find that there is a significant increase in denaturation in OA and this increased lower zone damage is accompanied by a significant loss of type II collagen compared to normal cartilage. When, however, we compare damage to the upper zone in normal and OA cartilages, we find that the more pronounced collagen denaturation observed in the upper layers of OA cartilage is not significantly different from the level of denaturation in the same zone of normal cartilage. This result is probably due to the fact that denaturation in the upper zone was very variable within the normal group (one specimen in particular), which may reflect early, pre-clinical, OA-like changes in normal cartilage (see Fig. 18). Indications from preliminary studies are that there is less denaturation in younger (skeletally mature) cartilages, suggesting that type II collagen denaturation may in part be a function of ageing. This may lead to excessive degenerative changes in some cases (A. Hollander, T. Heathfield and A.R. Poole, unpublished observations).

These findings on sites of damage to type II collagen confirm and extend our earlier work, using an immunohistochemical approach coupled with a polyclonal rabbit antiserum to epitopes in the CB8 and CB11 peptides of type II collagen. Those studies also revealed that there is increased damage in OA cartilage, which is first observed in the upper region (1). In separate immunohistochemical studies with monoclonal antibody COL2-3/4m we confirm that denaturation in normal and OA cartilages usually starts at and close to the articular surface and progressively extends down into the cartilage with increasing Mankin grade (A.P. Hollander, I. Pidoux, R. Bourne, C. Rorabeck and A.R. Poole, manuscript in preparation).

The mechanisms responsible for the denaturation (unwinding) of the triple helix have not been definitively identified. These studies are currently in progress. But the only known proteinase capable of causing unwinding of the triple helix of type II collagen is interstitial collagenase (matrix metalloproteinase-1; MMP-1). This cleaves the triple helix between residues 906 and 907 to produce the characteristic 1/4 and 3/4 pieces shown here, of which our monoclonal antibody recognizes the 3/4 piece. The use of antibody COL2-3/4m will hopefully assist us in identifying any other cleavage sites within the collagen molecule that may accompany the unwinding of the triple helix. Suffice it to say that the unwinding of the triple helix is not accompanied by such rapid secondary cleavage as to remove all α-chain fragments bearing the CB11B epitope and render them non-detectable. Such secondary trimming can occur by proteinases such as the 72kD and 92kD gelatinases, the expression of which are increased in OA cartilage as well as collagenase (15,11,20).

The fact that most (88.4%) of the denatured collagen extracted by α-chymotrypsin is not extractable with 4M guanidinium chloride indicates that it does not represent newly synthesized collagen. Rather, it reflects

denatured collagen bound to the cartilage matrix. Previously we showed that antibodies of the kind described here react primarily with denatured collagen fibrils (21). Thus based on those earlier studies, the majority of the denatured α-chains extracted with α-chymotrypsin most likely reflects denaturation of type II collagen present within cross-linked fibrils, often remote from chondrocytes. If the guanidinium chloride extractable pool represents denatured, newly synthesized collagen then the amount of this which is present in the cartilage accounts for a very small proportion of the denatured collagen. Studies are in progress to examine this question further.

Until we developed this new methodology, unwinding of the triple helix of type II collagen could not be detected in situ. To accurately quantitate degradation of collagen fibrils in cartilage it was necessary to develop an assay based on the use of a monoclonal antibody to a defined epitope of known location and which can be extracted intact, using selected proteinases to extract it first from non-helical α-chains and then from helical molecules. We identified and synthesized such an epitope located in the CB11 peptide and produced a monoclonal antibody to the synthetic peptide. The epitope is recognized in denatured but not native type II collagen α-chains. This has made possible development of an immunoassay, used in these studies and the detection of total type II collagen. Monoclonal COL2-3/4m cross-reacts with denatured type II collagen from all mammalian species so far studied (including mouse), indicating that the epitope contained within peptide α1(II)-CB11B is highly conserved. Importantly, binding of the antibody to the collagen α-chain is not dependent on hydroxylation of "Y" the position proline residues in the repeating Gly-X-Y triplet. This is important since it is possible that the extent of hydroxylation could change in pathological situations. Also, the epitope does not contain any potential cross-linking residues. Antibody COL2-3/4m cross-reacts with the α3(XI)

chain, which is considered to be the same gene product as the α1(II)-chain, although it is more heavily glycosylated (23,24). This cross-reactivity should not interfere with the quantitation of native or denatured type II collagen since there is only one α-3(XI)-chain per molecule of type XI collagen, which represents no more than 1% of total collagen α-chains in cartilage (25). The antibody does not cross-react with any other collagen or protein examined. Moreover, the epitope sequence is not present in any other known protein sequence.

Continuation of these studies should permit an improved understanding of the mechanisms involved in the denaturation of type II collagen in arthritis and may lead to a means of regulating this pathogenic process.

EXAMPLE 14

ENHANCED DAMAGE TO TYPE II COLLAGEN IN NORMAL ADULT HUMAN L5-S1 INTERVERTEBRAL DISCS COMPARED TO ARTICULAR CARTILAGE FROM SAME INDIVIDUALS

SUMMARY

Although there is histological evidence of collagen degradation in ageing intervertebral discs, these changes have not previously been quantitated, nor compared to articular cartilages. We have used a new, recently described immunochemical assay to measure the content of denatured and total type II collagen in macroscopically normal adult annulus fibrosus (AF) and nucleus pulposus (NP) of the L5-S1 intervertebral disc and in macroscopically normal articular cartilage obtained at autopsy from 7 individuals, including 1 male and 6 females, aged 33-76 years. The % denaturation of triple helical type II collagen was significantly higher in both AF (5.1 ± 6.6%) and NP (3.6 ± 1.7%) compared to cartilage (1.6 ± 1.0%). There was no significant difference in the levels of denaturation in AF compared to NP. There was also no significant difference in the total type II collagen content of AF (94.8 ± 21.6μg/mg wet weight) and NP (82.5 ± 26.9μg/mg wet weight) but these levels were

significantly lower than in articular cartilage (188.3 ± 36.5µg/mg wet weight). However, type II collagen accounted for only 64% of total collagen (measured as hydroxyproline) in the AF compared to 82% in the NP and 94% in articular cartilage. The glycosaminoglycan content of NP (80.2 ± 19.5µg/mg) was higher than in AF (58.3 ± 12µg/mg) or articular cartilage (48.4 ± 13.3µg/mg). In the NP there was a significant correlation ($r=0.78$, $P<0.05$) between the proteoglycan and type II collagen concentrations. These results demonstrate, for the first time, increased damage to type II collagen in the adult intervertebral disc compared to articular cartilage. This may have relevance to the degenerative changes which are commonly observed in the intervertebral disc with ageing.

There are 23 intervertebral discs in the adult human spine and each one is structurally characterized by 2 integrated tissues: the nucleus pulposus (NP) and the annulus fibrosus (AF). The function of these discs is to provide a deformable space between the vertebrae, which allows flexibility of the spine whilst providing resistance to compressive forces (26).

There is good documentation of the gross morphological changes in the intervertebral disc with age and in disease, including the formation of splits and clefts in the NP, tearing of the AF, marginal osteophyte formation and an overall thinning of the disc which is seen as a loss of disc height (27,28). Histological changes have also been observed, including a loss of staining for proteoglycan and increased damage to collagen fibrils (29). However, biochemical analyses of these changes have been limited to a few studies in which total content of water, collagen and proteoglycan (31,31), or the relative proportion (32) and distribution (33) of different collagens, have been measured. In human intervertebral disc, the NP contains collagen types II, VI and XI whilst the AF contains collagen types I, II, III, V, VI and XI (34). Type IX collagen is found in the AF and NP of intervertebral

disc from some species, but has not been reported in human disc tissue (33). Collagen types I and II together account for about 80% of the total collagen in intervertebral disc (34). The relative proportions of collagen types I and II in human intervertebral disc does not change with age (32). In the NP more than 85% of the collagen is type II, whereas in the AF as a whole only 50-65% is type II, although there is a gradual change from mainly type I on the outside of the AF to mainly type II on the inside(32). Absolute concentrations of each collagen have not previously been measured and so there is no data available to indicate if the observed differences in the relative proportions in the AF and NP represent differences in the content of type I, type II or both collagens. The total collagen content, measured as hydroxyproline, is approximately twice as high on a μg/mg dry weight basis in the AF as in the NP of the human intervertebral disc (26,35). The proteoglycans in intervertebral disc include the large aggregating proteoglycan, aggrecan, as well as a relatively high proportion of non-aggregating proteoglycans (36). About 85% of the proteoglycans isolated from hyaline cartilage are able to interact with hyaluronate whereas only 30-40% of proteoglycans isolated from mature human intervertebral discs are able to interact (37).

Studies comparing the biochemical changes of ageing human intervertebral discs with changes in their gross morphology or magnetic resonance imaging profiles have shown that damage to the disc correlates with a decreased proteoglycan concentration (30,31). There was either no correlation with total collagen, measured as hydroxyproline, or possibly an increased collagen concentration with increased damage to the disc in these studies. However, histological analysis has shown clearly that there is increased fraying and fragmentation of collagen in ageing human intervertebral disc (29). Therefore measurements of collagen denaturation and fragmentation may correlate

better with morphological changes than does total hydroxyproline content.

In these studies, we found that in rheumatoid arthritis cleavage of type II collagen is first observed in territorial regions around cells in the deep zone closest to subchondral bone, as well as in the superficial zone, at the articular surface and at the pannus-cartilage junction. In advanced disease, extensive degradation was observed throughout the cartilage matrix. In ageing and osteoarthritis, cleavage of type II collagen was first observed in the superficial and upper-mid zones in specimens with a low Mankin grade. In more advanced osteoarthritis (higher Mankin grade specimens) the cleavage extended through the mid zone into the deep zone (38,39).

In this study we have used this new methodology to measure the content of denatured and total type II collagen in the AF and NP of the adult human L5-S1 intervertebral disc compared to articular cartilage from the same individuals. We show for the first time that type II collagen in the adult disc is more degraded than type II collagen in the corresponding articular cartilage.

MATERIALS AND METHODS

SOURCES OF TISSUES.

Human knee articular cartilage and intervertebral disc tissue were obtained at autopsy (within 15h post-mortem) from 7 adults who were without macroscopic signs of arthritic/joint/spinal abnormalities. Table IX shows the age, sex and cause of death of each individual.

Full-depth specimens of articular cartilage were taken from the anterior (loaded) region of the femoral condyles (medial and lateral compartments) of each individual. Specimens of the L5-S1 intervertebral disc from the same individuals were obtained by removing a wedge-shaped block of tissue from the anterior region of the AF, extending into the NP, as shown in Fig. 21. The disc specimens all had normal gross morphology for their age and were classified as grades II or III (out of a maximum of V) by the

morphological grading system of Thompson et al (27). All tissue samples were wrapped in plastic immediately after collection to maintain humidity and they were maintained at room temperature prior to immediate processing and weighing.

PREPARATION OF TISSUE.

Three full depth adjacent blocks of tissue, each approximately 3mm X 5mm, were cut from the articular cartilage and from standardized regions of the AF and NP specimens (Fig. 21) using a scalpel. The wet weight of each block of tissue was immediately recorded. Extraction of collagens was initiated without storage. One block from each tissue site was used for the extraction of denatured and native type II collagens and the subsequent assay of the α1(II)-CB11B epitope and of hydroxyproline. A second, adjacent block was used for the extraction and assay of proteoglycans and the third block was prepared for immunohistochemical analysis of denatured type II collagen.

EXTRACTION OF DENATURED AND NATIVE TYPE II COLLAGENS AND DETERMINATION OF TOTAL TYPE II COLLAGEN CONTENT.

Tissue blocks were routinely extracted, first with α-chymotrypsin (to solubilize denatured collagen) and then proteinase K (to digest remaining native collagen α-chains), as previously described. Briefly, the tissue blocks (30-40mg wet weight) were distributed individually into Eppendorf tubes. To each tube was added 500µl of 1mg/ml α-chymotrypsin in 0.5M Tris, pH 7.2 containing 1mM iodoacetamide, 1mM EDTA and 10µg/ml pepstatin-A (all from Sigma Chemical Co., St. Louis, MO). The samples were incubated overnight at 37°C. The α-chymotrypsin was inhibited by addition of 200µl per tube of 0.4 mg/ml TPCK in 0.5M Tris containing 4% v/v ethanol. The α-chymotrypsin extract was separated from the residue. Each residue was then digested with 500µl of 1mg/ml proteinase K in Tris containing the same protease inhibitors as described above. After digestion overnight at 56°C, no residue remained. The proteinase K was inactivated and the extracted collagen

fully denatured by boiling the samples for 10 min. In order to measure any native collagen extracted by α-chymotrypsin, a 300µl aliquot of each α-chymotrypsin extract was mixed with 100µl of the 1mg/ml proteinase K solution and incubated and then boiled as described for cartilage residues (see above). The TPCK-inhibited α-chymotrypsin and boiled proteinase K had no effect on the immunoassay.

The tissue extracts were assayed for hydroxyproline by a colorimetric method (40). The total collagen content (all types) was estimated on the basis that the hydroxyproline content of the collagens is equivalent to 10% of the weight of each α-chain (41).

Tissue extracts were assayed for denatured collagen as previously described in the above examples, using the α1(II)-CB11B peptide as a standard. Briefly, the samples were preincubated with antibody COL2-3/4m overnight at 37°C, transferred to ELISA plates coated with heat denatured type II collagen and incubated for a further 30 min at room temperature. Inhibition by the samples of antibody-binding to the heat denatured collagen was detected using an alkaline phosphatase labelled goat anti-mouse immunoglobulin second-step antibody and alkaline phosphatase substrate.

The total type II collagen content of each tissue specimen was calculated on the basis of the measured concentration of α1(II)-CB11B, as previously described.

EXTRACTION AND ASSAY OF PROTEOGLYCANS.

Blocks of tissue were distributed individually into Eppendorf tubes and digested directly with proteinase K, as described above. Aliquots of the digested tissues were assayed for glycosaminoglycans by a micro-colorimetric assay using dimethylmethylene blue, as previously described (41).

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Tissue blocks were mounted in OCT embedding media (Miles Laboratories, Naperville, IL), frozen and 6µm thick sections were cut at -20°C using a Tissue-Tek II cryostat

(Miles Laboratories). Sections were picked up on glass microscope slides precoated with aminoalkylsilane as described by Henderson(42), to ensure complete adherence of the tissue sections to the slides. This is very important in these studies since our monoclonal antibody will detect denatured type II collagen at the surfaces or edges of the section. Thus complete adherence is essential to ensure uniformity of immunoreactivity.

Sections were either immediately frozen and stored at -20°C or used immediately. Similar results were obtained in either case. The sections were fixed for 5 min in 4% formaldehyde, freshly prepared from paraformaldehyde in phosphate buffered saline (PBS) (43) and washed in several changes of PBS for 15 min. These and subsequent manipulations were at room temperature. Unreactive aldehyde groups were blocked with 1%w/v normal pig serum in PBS for 15 min. Endogenous peroxidase activity was blocked by incubation of the sections with freshly prepared 0.5%v/v H_2O_2 in absolute methanol for 10 min followed by washing in PBS for 15 min. To enhance permeability of the tissue, sections were treated with chondroitinase ABC (ICN/Flow, Mississauga, ON) at 0.0125U/50µl per section in 0.1M Tris-acetate buffer, pH 7.6, for 90 min at 37°C in a humidified environment to remove chondroitin sulfate (44). After washing in PBS for 15 min the sections were incubated with 0.2M EDTA in 50mM Tris, pH 7.6 for 1h at room temperature in order to ensure removal of any calcific deposits in the specimens. They were then washed for 15 min with PBS containing 0.1%w/v BSA.

Sections were incubated for 30 min at room temperature with a humidified chamber with 50µl/section of monoclonal antibody COL2-3/4m ascitic fluid (diluted 1/100) or control, non-immune ascitic fluid, each diluted in PBS containing 1% w/v BSA. An additional specific control was prepared by absorbing monoclonal antibody COL2-3/4m before use with 100µg/ml of peptide α1(II)-CB11B, or an unrelated peptide of the same length and concentration, at 37°C for

1h and centrifugation before use to remove any precipitate. Sections were washed 3 times for 10 min each with PBS containing 0.1%w/v BSA. A biotin-streptavidin detection system (Amersham Corp., Arlington Heights, IL) was used consisting of pig $F(ab')_2$ anti-mouse Ig labelled with biotin and peroxidase-conjugated streptavidin as the indicator. The biotinylated pig anti-mouse $F(ab')_2$ (prepared and labelled in this laboratory) was used at a dilution of 1/20 in PBS with 1%w/v BSA at 50µl/section for 30 min. After washing with azide-free PBS, the sections were treated with 50µl/section of the peroxidase-conjugated streptavidin at a dilution of 1/150 in azide-free PBS with 1%w/v BSA for 20 min at room temperature. The sections were washed twice for 10 min in azide-free PBS, post-fixed by incubating with 2%v/v gluteraldehyde in azide-free PBS and then stored in 50mM Tris, pH 7.6, for 24h. The peroxidase reaction was performed with copper-H_2O_2/silver intensification of the nickel-diaminobenzidine end-product of the peroxidase reaction, as previously described (45).

STATISTICAL ANALYSIS.

Significance of differences in the content of collagen, hydroxyproline and proteoglycan in the AF and NP compared to paired cartilage from each individual case were tested using the 2-tailed paired t-test. Correlations were made using spearman rank regression analyses.

RESULTS

CONTENTS OF TOTAL TYPE II COLLAGEN AND HYDROXYPROLINE.

The total type II collagen content determined by the α1(II)-CB11B assay and expressed as µg/mg wet weight of tissue was significantly lower in both the AF and NP than in the articular cartilage and the same trend was noted in all 7 cases (Fig. 22). The mean (SD) values for cartilage, AF and NP were 188.3 (36.5) µg/mg, 94.8 (21.6) µg/mg and 82.5 (26.9) µg/mg respectively. Surprisingly, the total type II collagen concentration in the AF was not significantly different to the NP.

The hydroxyproline content expressed as µg/mg wet weight of tissue was also significantly lower in both the AF and NP compared to the cartilage. There was a trend towards higher levels of hydroxyproline in the AF than in the NP, seen in 6 of 7 cases (Fig. 23), but this difference was not significant. The mean (SD) values for cartilage, AF and NP were 20.1 (1.0) µg/mg, 15.0 (3.2) µg/mg and 10.9(4.6) µg/mg respectively.

The ratio of µg type II collagen:µg hydroxyproline was lower in the AF than in the cartilage of all 7 cases and this difference was significant (Fig. 24), demonstrating that a significant proportion of collagen in the AF is not type II. In contrast, there was no significant difference in this ratio in the NP compared to cartilage (Fig. 24). The mean (SD) values for cartilage, AF and NP were 9.4 (1.9), 6.4 (0.7) and 8.2 (3.0) respectively. Assuming that the hydroxyproline content of the fibrillar collagens is 10% of the weight of each α-chain (14), the ratios indicate that type II collagen accounts for approximately 82% of the total collagen in NP, but only 64% of total collagen in the AF of the L5-S1 intervertebral disc, whereas in the articular cartilage type II collagen represents 94% of the total collagen content. This data is similar to that of Eyre and Muir, who found that in the adult human intervertebral disc over 85% of the collagen in NP was type II and in articular cartilage over 95% was type II, whereas in AF type II collagen represented 50-65% of total collagen (32).

These results show that although the absolute concentration of type II collagen is essentially the same in the AF as in the NP, its proportion relative to other collagens varies. Therefore, the previously reported differences in the ratio of type I to type II collagens in these 2 regions of the disc (32) must be accounted for by a higher content of type I collagen rather than a lower content of type II collagen in the AF.

CONTENTS OF DENATURED TYPE II COLLAGEN IN CARTILAGE AND INTERVERTEBRAL DISC.

The % denatured type II collagen content determined by the α1(II)-CB11B assay was always higher in both the AF and NP than in the corresponding articular cartilage of all 7 cases (A-G). As a group, these differences were significant ($p<0.01$; Fig. 25). There was no significant difference in the content of denatured type II collagen in the AF compared to NP. More denaturation was noted in the AF than the NP in 3 of the cases, the reverse trend in 1 case and similar levels of denaturation in the AF and NP in 3 cases. The mean (SD) values for cartilage, AF and NP were 1.6 (1.0) %, 5.1 (6.6) % and 3.6 (1.7) % respectively. These results clearly show that type II collagen in adult, morphologically normal L5-S1 intervertebral disc (AF and NP) is more damaged than in the articular cartilage of the same individual.

CONTENTS OF TYPE II COLLAGEN RELATIVE TO PROTEOGLYCANS.

The proteoglycan content, measured as GAG and expressed as μg/mg wet weight of tissue, was significantly higher in the NP than in the articular cartilage of all 7 cases and this difference was significant (Fig. 26). There was no significant difference in the GAG content of the AF compared to cartilage (Fig. 26). The mean (SD) values of GAG content for cartilage, AF and NP were 48.4 (13.3) μg/mg, 58.3 (12.0) μg/mg and 80.2 (19.5) μg/mg respectively. This data is in agreement with previous studies showing a higher proteoglycan concentration in the NP than the AF (26).

Figure 27 shows correlations between the concentrations of type II collagen and GAG. There was no significant trend in this relationship in either cartilage (Fig. 27a; $r=0.41$, NS) or AF (Fig. 27b; $r=0.68$, NS). However, in NP there was a significant trend towards lower concentrations of type II collagen in specimens with a low concentration of proteoglycan (Fig.27c; $r=0.78$, $p<0.05$). The results show that although these intervertebral discs

all had a normal gross morphology (see above), some of them nevertheless exhibit biochemical signs of matrix deterioration, demonstrated as a loss of proteoglycan accompanied by a loss of type II collagen in the NP.

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Staining of the articular cartilage with monoclonal antibody COL2-3/4m was either absent or limited to diffuse staining in the superficial zone (Fig. 28A). In contrast, there was intense staining throughout the matrix and in pericellular regions of both the AF and NP (Figs. 28B and C). Specimens treated with non-immune ascitic fluid or COL2-3/4m pre-absorbed with peptide α1(II)-CB11B showed minimal staining (Figs. 28D and E).

DISCUSSION

The collagen fibrillar network is responsible for the tensile strength of cartilages and related tissues. Histological evidence of increased damage to collagen fibrils in adult intervertebral discs (29) has been reported in previous studies and this is functionally related to structural damage in discs, which is very prevalent in adults (28). It is difficult to distinguish this ageing phenomenon from changes associated with clinical diseases of the spine. In contrast, other studies have failed to demonstrate any significant quantitative changes in the integrity of the fibrillar network or its collagen content in the AF or NP with age or with degeneration (30,31). Using newly developed methodology we show here, for the first time, that the damage to type II collagen in morphologically normal adult human L5-S1 intervertebral disc is significantly greater than in paired, normal articular cartilage. The increased denaturation of type II collagen in the disc was similar to the damage seen in articular cartilage from patients with osteoarthritis or rheumatoid arthritis (46), suggesting that there is considerable potential for collagen degradation in clinically normal adult disc. Damage was much more pronounced in some discs than in others and in some cases the damage was more

pronounced in the AF than in the NP. The extensive damage revealed by immunohistochemical staining with monoclonal antibody COL2-3/4m throughout the AF and NP also contrasts to the more limited and restricted staining seen in articular cartilage.

We also show that the lower proportion type II collagen relative to other collagens in the AF, demonstrated here and previously (32), is probably due to a higher content of type I collagen in the AF, in view of the similar type II collagen contents in the AF and NP. Confirmation of this finding must await the development of methodology for measuring denatured and total type I collagen. These studies are in progress.

We show that in the NP a loss of proteoglycans is associated with a loss of type II collagen in discs with normal gross morphology. These changes are indicative of alterations within the structure of the disc matrix that would be expected to be determinal to disc function. Indeed, Pearce et al (30) have reported that a decrease in proteoglycan content accompanies disc degeneration and may precede overt morphological damage. Similarly, it is likely that the increased level of type II collagen damage observed in the morphologically normal disc in this study may also be a prelude to degeneration.

In this study we have chosen to focus on the extent of damage to type II collagen in normal adult intervertebral disc compared to normal articular cartilage. We are currently investigating the relationship of ageing and disease to damage of type II collagen in degenerated disc. Such analysis may help to identify and distinguish pathological changes from ageing phenomena at a biochemical level, so improving our understanding of the degeneration of the intervertebral disc that occurs in the adult.

Table V Patient details

Disease group	n	Mean age in years	Age range	% Male-Female	Median Mankin grade	Range of Mankin grades
Normal	8	56	28-81	50:50	2	0-6
OA	16	69	57-84	57:43	5	2-10

Table VI Clostridial collagenase cleaves the α1(II)-CB11B epitope

Sample	Treatment	[α1(II)-CB11B] by immunoassay (μg/ml)
Tris	Bacterial collagenase	0.85
HDC	Tris/$CaCl_2$	3.80
HDC	Clostridial collagenase	0.84
α1(II)-CB11B	Tris/$CaCl_2$	4.44
α1(II)-CB11B	Clostridial collagenase	0.83

HDC and α1(II)-CB11B were dissolved in Tris containing 5mM $CaCl_2$ and 14 U/ml chromatographically purified bacterial collagenase. Control tubes contained HDC or α1(II)-CB11B in Tris with 5mM $CaCl_2$ but no bacterial collagenase or bacterial collagenase in Tris with 5mM $CaCl_2$ but no HDC or α1(II)-CB11B. The samples were incubated overnight at 37°C and the bacterial collagenase was inactivated by addition of EDTA to a final concentration of 100mM (this concentration was required to ensure maximal inhibition of the proteinase).

Table VII The inhibition observed in cartilage extracts is lost on treatment with Clostridial collagenase

Sample	Proteinase Digestion	[α1(II)-CB11B] by immunoassay (µg/ml)	
		Digested sample treated with Tris/$CaCl_2$	Digested sample treated with Tris/$CaCl_2$ + collagenase
Tris	None	0.00	0.46
OA-(i)	α-chymotrypsin	1.39	0.47
OA-(ii)	α-chymotrypsin	3.22	0.45
OA-(iii)	α-chymotrypsin	2.11	0.43
OA-(iv)	α-chymotrypsin	3.53	0.48
OA-(v)	α-chymotrypsin	2.86	0.47
OA-(i)	Proteinase K	53.4	0.49
OA-(ii)	Proteinase K	93.2	0.47
OA-(iii)	Proteinase K	82.5	0.47
OA-(iv)	Proteinase K	148.9	0.40
OA-(v)	Proteinase K	61.7	0.56

OA cartilages (i-v) were extracted/digested with α-chymotrypsin and proteinase K. The digested samples were then treated with Tris/5mM$CaCl_2$ with or without 14U/ml Clostridial collagenase at 37°C. Clostridial collagenase activity was inhibited with 100mM EDTA. The samples were assayed for α1(II)-CB11B.

Table VIII Total and denatured collagen in different depths of normal and OA cartilage

Disease group	[Total type II collagen] (µg/mg)		% Denatured type II collagen	
	Zones		Zones	
	Upper	Lower	Upper	Lower
Normal	139.9	179.5	2.42	1.12
OA	92.4^{NS}	128.9*	3.2^{NS}	2.2**

Zones are 1mm thick. Values are the mean result for cartilage from 5 normal and 8 OA patients. NS=not significant, *$p<0.03$; **$p<0.02$ v. normal cartilage; Mann-Whitney U-test. Results for the individual patients and statistical comparisons of data for upper versus lower zones in each patient group are shown in Fig. 9.

Table IX Details of individual cases

AUTOPSY CASE	AGE (YEARS)	SEX	CAUSE OF DEATH
A	76	Male	Cardiac Arrest
B	72	Female	Brain Tumor
C	33	Female	Brain Tumor
D	67	Female	Gastric Carcinoma
E	60	Female	Aspiration
F	72	Female	Lymphoma
G	39	Female	Breast Carcinoma

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(ii) TITLE OF INVENTION: IMMUNOASSAY FOR THE MEASUREMENT OF COLLAGEN CLEAVAGE IN CARTILAGE

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1418 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Human Type II Collagen

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Val Leu Leu Thr Leu Leu
1 5 10 15

Val Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Val Arg Gln Pro Gly
20 25 30

Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile Lys Asp Ile Val Gly
35 40 45

Pro Lys Gly Pro Pro Gly Pro Gln Gly Pro Ala Gly Glu Gln Gly Pro
50 55 60

Arg Gly Asp Arg Gly Asp Lys Gly Glu Lys Gly Ala Pro Gly Pro Arg
65 70 75 80

Gly Arg Asp Gly Glu Pro Gly Thr Leu Gly Asn Pro Gly Pro Pro Gly
85 90 95

Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly Asn Phe Ala Ala
100 105 110

Gln Met Ala Gly Gly Phe Asp Glu Lys Ala Gly Gly Ala Gln Leu Gly
115 120 125

Val Met Gln Gly Pro Met Gly Pro Met Gly Pro Arg Gly Pro Pro Gly
130 135 140

Pro Ala Gly Ala Pro Gly Pro Gln Gly Phe Gln Gly Asn Pro Gly Glu
145 150 155 160

Pro Gly Glu Pro Gly Val Ser Gly Pro Met Gly Pro Arg Gly Pro Pro
165 170 175

Gly Pro Pro Gly Lys Pro Gly Asp Asp Gly Glu Ala Gly Lys Pro Gly
180 185 190

Lys Ala Gly Glu Arg Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly Phe
195 200 205

Pro Gly Thr Pro Gly Leu Pro Gly Val Lys Gly His Arg Gly Tyr Pro
210 215 220

Gly Leu Asp Gly Ala Lys Gly Glu Ala Gly Ala Pro Gly Val Lys Gly
225 230 235 240

Glu Ser Gly Ser Pro Gly Glu Asn Gly Ser Pro Gly Pro Met Gly Pro
245 250 255

Arg Gly Leu Pro Gly Glu Arg Gly Arg Thr Gly Pro Ala Gly Ala Ala
260 265 270

Gly Ala Arg Gly Asn Asp Gly Gln Pro Gly Pro Ala Gly Pro Pro Gly
275 280 285

Pro Val Gly Pro Ala Gly Gly Pro Gly Phe Pro Gly Ala Pro Gly Ala
290 295 300

Lys Gly Glu Ala Gly Pro Thr Gly Ala Arg Gly Pro Glu Gly Ala Gln
305 310 315 320

Gly Pro Arg Gly Glu Pro Gly Thr Pro Gly Ser Pro Gly Pro Ala Gly
325 330 335

Ala Ser Gly Asn Pro Gly Thr Asp Gly Ile Pro Gly Ala Lys Gly Ser
340 345 350

Ala Gly Ala Pro Gly Ile Ala Gly Ala Pro Gly Phe Pro Gly Pro Arg
355 360 365

Gly Pro Pro Asp Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly
370 375 380

Gln Thr Gly Lys Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro
385 390 395 400

Lys Gly Glu Pro Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala
405 410 415

Gly Glu Glu Gly Lys Arg Gly Ala Arg Gly Glu Pro Gly Gly Val Gly
420 425 430

Pro Ile Gly Pro Pro Gly Glu Arg Gly Ala Pro Gly Asn Arg Gly Phe
435 440 445

Pro Gly Gln Asp Gly Leu Ala Gly Pro Lys Gly Ala Pro Gly Glu Arg
450 455 460

Gly Pro Ser Gly Leu Ala Gly Pro Lys Gly Ala Asn Gly Asp Pro Gly
465 470 475 480

Arg Pro Gly Glu Pro Gly Leu Pro Gly Ala Arg Gly Leu Thr Gly Arg
485 490 495

Pro Gly Asp Ala Gly Pro Gln Gly Lys Val Gly Pro Ser Gly Ala Pro
500 505 510

Gly Glu Asp Gly Arg Pro Gly Pro Pro Gly Pro Gln Gly Ala Arg Gly
515 520 525

Gln Pro Gly Val Met Gly Phe Pro Gly Pro Lys Gly Ala Asn Gly Glu
530 535 540

Pro Gly Lys Ala Gly Glu Lys Gly Leu Pro Gly Ala Pro Gly Leu Arg
545 550 555 560

Gly Leu Pro Gly Lys Asp Gly Glu Thr Gly Ala Glu Gly Pro Pro Gly
565 570 575

Pro Ala Gly Pro Ala Gly Glu Arg Gly Glu Gln Gly Ala Pro Gly Pro
580 585 590

Ser Gly Phe Gln Gly Leu Pro Gly Pro Pro Gly Pro Pro Gly Glu Gly
595 600 605

Gly Lys Pro Gly Asp Gln Gly Val Pro Gly Glu Ala Gly Ala Pro Gly
610 615 620

Leu Val Gly Pro Arg Gly Glu Arg Gly Phe Pro Gly Glu Arg Gly Ser
625 630 635 640

Pro Gly Ala Gln Gly Leu Gln Gly Pro Arg Gly Leu Pro Gly Thr Pro
645 650 655

Gly Thr Asp Gly Pro Lys Gly Ala Ser Gly Pro Ala Gly Pro Pro Gly
660 665 670

Ala Gln Gly Pro Pro Gly Leu Gln Gly Met Pro Gly Glu Arg Gly Ala
675 680 685

Ala Gly Ile Ala Gly Pro Lys Gly Asp Arg Gly Asp Val Gly Glu Lys
690 695 700

Gly Pro Glu Gly Ala Pro Gly Lys Asp Gly Gly Arg Gly Leu Thr Gly
705 710 715 720

Pro Ile Gly Pro Pro Gly Pro Ala Gly Ala Asn Gly Glu Lys Gly Glu
725 730 735

Val Gly Pro Pro Gly Pro Ala Gly Ser Ala Gly Ala Arg Gly Ala Pro
740 745 750

Gly Glu Arg Gly Glu Thr Gly Pro Pro Gly Thr Ser Gly Ile Ala Gly
755 760 765

Pro Pro Gly Ala Asp Gly Gln Pro Gly Ala Lys Gly Glu Gln Gly Glu
770 775 780

Ala Gly Gln Lys Gly Asp Ala Gly Ala Pro Gly Pro Gln Gly Pro Ser
785 790 795 800

Gly Ala Pro Gly Pro Gln Gly Pro Thr Gly Val Thr Gly Pro Lys Gly
805 810 815

Ala Arg Gly Ala Gln Gly Pro Pro Gly Ala Thr Gly Phe Pro Gly Ala
820 825 830

Ala Gly Arg Val Gly Pro Pro Gly Ser Asn Gly Asn Pro Gly Pro Pro
835 840 845

Gly Pro Pro Gly Pro Ser Gly Lys Asp Gly Pro Lys Gly Ala Arg Gly
850 855 860
Asp Ser Gly Pro Pro Gly Arg Ala Gly Glu Pro Gly Leu Gln Gly Pro
865 870 875 880

Ala Gly Pro Pro Gly Glu Lys Gly Glu Pro Gly Asp Asp Gly Pro Ser
885 890 895

Gly Ala Glu Gly Pro Pro Gly Pro Gln Gly Leu Ala Gly Gln Arg Gly
900 905 910

Ile Val Gly Leu Pro Gly Gln Arg Gly Glu Arg Gly Phe Pro Gly Leu
915 920 925

Pro Gly Pro Ser Gly Glu Pro Gly Gln Gln Gly Ala Pro Gly Ala Ser
930 935 940

Gly Asp Arg Gly Pro Pro Gly Pro Val Gly Pro Pro Gly Leu Thr Gly
945 950 955 960

Pro Ala Gly Glu Pro Gly Arg Glu Gly Ser Pro Gly Ala Asp Gly Pro
965 970 975

Pro Gly Arg Asp Gly Ala Ala Gly Val Lys Gly Asp Arg Gly Glu Thr
980 985 990

Gly Ala Val Gly Ala Pro Gly Ala Pro Gly Pro Pro Gly Ser Pro Gly
995 1000 1005

Pro Ala Gly Pro Thr Gly Lys Gln Gly Asp Arg Gly Glu Ala Gly Ala
1010 1015 1020

Gln Gly Pro Met Gly Pro Ser Gly Pro Ala Gly Ala Arg Gly Ile Gln
1025 1030 1035 1040

Gly Pro Gln Gly Pro Arg Gly Asp Lys Gly Glu Ala Gly Glu Pro Gly
1045 1050 1055

Glu Arg Gly Leu Lys Gly His Arg Gly Phe Thr Gly Leu Gln Gly Leu
1060 1065 1070

Pro Gly Pro Pro Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala
1075 1080 1085

Gly Pro Ser Gly Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly
1090 1095 1100

Lys Asp Gly Ala Met Gly Ile Pro Gly Pro Ile Gly Pro Pro Gly Pro
1105 1110 1115 1120

Arg Gly Arg Ser Gly Glu Thr Gly Pro Ala Gly Pro Pro Gly Asn Pro
1125 1130 1135

Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Gly Ile Asp Met Ser
1140 1145 1150

Ala Phe Ala Gly Leu Gly Pro Arg Glu Lys Gly Pro Asp Pro Leu Gln
1155 1160 1165

Tyr Met Arg Ala Asp Gln Ala Ala Gly Gly Leu Arg Gln His Asp Ala
1170 1175 1180

Glu Val Asp Ala Thr Leu Lys Ser Leu Asn Asn Gln Ile Glu Ser Ile
1185 1190 1195 1200

Arg Ser Pro Glu Gly Ser Arg Lys Asn Pro Ala Arg Thr Cys Arg Asp
1205 1210 1215

Leu Lys Leu Cys His Pro Glu Trp Lys Ser Gly Asp Tyr Trp Ile Asp
1220 1225 1230

Pro Asn Gln Gly Cys Thr Leu Asp Ala Met Lys Val Phe Cys Asn Met
1235 1240 1245

Glu Thr Gly Glu Thr Cys Val Tyr Pro Asn Pro Ala Asn Val Pro Lys
1250 1255 1260

Lys Asn Trp Trp Ser Ser Lys Ser Lys Glu Lys Lys His Ile Trp Phe
1265 1270 1275 1280

Gly Glu Thr Ile Asn Gly Gly Phe His Phe Ser Tyr Gly Asp Asp Asn
1285 1290 1295

Leu Ala Pro Asn Thr Ala Asn Val Gln Met Thr Phe Leu Arg Leu Leu
1300 1305 1310

Ser Thr Glu Gly Ser Gln Asn Ile Thr Tyr His Cys Lys Asn Cys Ile
1315 1320 1325

Ala Tyr Leu Asp Glu Ala Ala Gly Asn Leu Lys Lys Ala Leu Leu Ile
1330 1335 1340

Gln Gly Ser Asn Asp Val Glu Ile Arg Ala Glu Gly Asn Ser Arg Phe
1345 1350 1355 1360

Thr Tyr Thr Ala Leu Lys Asp Gly Cys Thr Lys His Thr Gly Lys Trp
1365 1370 1375

Gly Lys Thr Val Ile Glu Tyr Arg Ser Gln Lys Thr Ser Arg Leu Pro
1380 1385 1390

Ile Ile Asp Ile Ala Pro Met Asp Ile Gly Gly Pro Glu Gln Glu Phe
1395 1400 1405

Gly Val Asp Ile Gly Pro Val Cys Phe Leu
1410 1415

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 279 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CB11 peptide of Type II Collagen

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly Arg Thr Gly Pro Ala Gly
1 5 10 15

Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln Pro Gly Pro Ala Gly Pro
20 25 30

Pro Gly Pro Val Gly Pro Ala Gly Gly Pro Gly Phe Pro Gly Ala Pro
35 40 45

Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly Ala Arg Gly Pro Glu Gly
50 55 60

Ala Gln Gly Pro Arg Gly Glu Pro Gly Thr Pro Gly Ser Pro Gly Pro
65 70 75 80

Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp Gly Ile Pro Gly Ala Lys
85 90 95

Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly Ala Pro Gly Phe Pro Gly
100 105 110

Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro
115 120 125

Lys Gly Gln Thr Gly Lys Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln
130 135 140

Gly Pro Lys Gly Glu Pro Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly
145 150 155 160

Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala Arg Gly Glu Pro Gly Gly
165 170 175

Val Gly Pro Ile Gly Pro Pro Gly Glu Arg Gly Ala Pro Gly Asn Arg
180 185 190

Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly Pro Lys Gly Ala Pro Gly
195 200 205

Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro Lys Gly Ala Asn Gly Asp
210 215 220

Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro Gly Ala Arg Gly Leu Thr
225 230 235 240

Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly Lys Val Gly Pro Ser Gly
245 250 255

Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro Pro Gly Pro Gln Gly Ala
260 265 270

Arg Gly Gln Pro Gly Val Met
275

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CB11B peptide of Type II Collagen

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Cys Gly Lys Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro
1 5 10 15

Gly Pro Pro Gly Pro Gln Tyr
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:
(A) ORGANISM: CB11B/H peptide of Type II Collagen

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro Pro Gly Pro
1 5 10

CLAIMS:

1. A method for the determination of cartilage degradation by measuring the amount of unwound collagen present in a biological sample, said method comprising:
- contacting the biological sample with a monoclonal antibody which has the ability to bind to an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen; and
- detecting unwound collagen and fragments thereof bound to said monoclonal antibody.

2. A method according to claim 1, wherein the amount of unwound collagen detected is the amount of unwound type II collagen.

3. A method according to claim 1, wherein the amount of unwound collagen detected is the amount of unwound type XI collagen.

4. A method according to claim 1, wherein said monoclonal antibody has the ability to bind to an epitope on unwound type II collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical type II collagen.

5. A method according to claim 1, further comprising a preliminary step which comprises contacting said biological sample with a first enzyme having the ability to selectively cleave unwound collagen chains without degrading said epitope, and extracting the collagen fragments that react with said first enzyme from said biological sample to produce an enzyme extract that is contacting with the monoclonal antibody according to the method of claim 1.

6. A method according to claim 5, wherein said first enzyme is selected from the group consisting of chymotrypsin and trypsin.

7. A method according to claim 5, further comprising treating an aliquot of said enzyme extract and said biological sample that is substantially free of the enzyme extract to solubilize and unwind remaining native collagen contained in said aliquot and said biological sample, and contacting said aliquot and said biological sample with the monoclonal antibody according to the method of claim 1 without degrading said epitope.

8. A method according to claim 7, wherein said native collagen is solubilized and unwound by incubating said aliquot and said biological sample with a second enzyme having the ability to solubilize helical collagen from cartilage without cleaving the antibody-reacting epitope and by submitting said solubilized collagen to heat denaturation.

9. A method according to claim 8, wherein said second enzyme is proteinase K.

10. A method for the determination of cartilage degradation by quantitating the amount of unwound collagen, said method comprising:

- contacting a biological sample with an enzyme having the ability to selectively cleave unwound collagen chains in said biological sample into collagen fragments without cleaving an antibody-reactive epitope on said unwound collagen chains;
- extracting the collagen fragments that react with said enzyme from said biological sample to produce an enzyme extract;

- removing an aliquot from said extract and treating said aliquot to unwind any wound collagen contained therein;
- treating said biological sample to solubilize and unwind remaining native collagen contained therein without degrading the antibody reactive epitope;
- measuring the amount of unwound collagen present in said extract, said aliquot of said extract and said solubilized biological sample by separately contacting said extract, said aliquot and said biological sample with a monoclonal antibody which has the ability to bind an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen, and determining the amount of said unwound collagen and fragments thereof bound to said monoclonal antibody; and
- determining the amount of collagen that is unwound by comparing the immunological reaction of said monoclonal antibody to said extract said aliquot and said solubilized biological sample.

11. A method according to claim 10, wherein said biological sample is selected from a biological tissue and a biological fluid.

12. A method for measuring total collagen content in a biological sample, said method comprising:
- treating said biological sample to solubilize and unwind collagen contained therein without degrading an epitope that reacts with a monoclonal antibody; and
- measuring the amount of unwound collagen present in said solubilized biological sample by contacting said biological sample with the monoclonal antibody which has the ability to bind an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said

monoclonal antibody does not bind to native helical collagen, and determining the amount of said unwound collagen and fragments thereof bound to said monoclonal antibody.

13. A method according to claim 12, wherein said collagen is type II collagen.

14. A method according to claim 12, wherein said collagen is type XI collagen.

15. A use of a method according to claim 1, 10 or 12 to measure collagen degradation in osteoarthritic cartilage.

16. A use of a method according to claim 1, 10 or 12 to measure collagen degradation in intervertebral discs.

17. A kit for the measurement of cartilage degradation products in a biological sample, said kit comprising:

- a monoclonal antibody which has the ability to bind to an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen;
- a solid support for binding proteins; and
- a labelled antibody to measure the binding of said monoclonal antibody to said unwound collagen.

18. A kit according to claim 17, wherein said monoclonal antibody has the ability to bind to an epitope on unwound type II collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical type II collagen.

19. A kit according to claim 17, further comprising an enzyme having the ability to selectively cleave unwound

collagen chains in said biological sample without cleaving antibody-reactive epitope on said collagen chains to produce an extract comprising fragments of unwound collagen.

20. A kit according to claim 19, wherein said enzyme is chymotrypsin.

21. A kit according to claim 19, further comprising means for solubilizing native collagen from said biological sample to convert said native helical collagen to unwound collagen without degrading said epitope.

22. A kit according to claim 21, wherein said solubilizing means comprise an enzyme having the ability to solubilize helical collagen from cartilage without cleaving the antibody-reactive epitope.

23. A kit according to claim 22, wherein said enzyme is proteinase K.

24. A use of a kit according to claim 17, 18, 19, 20, 21, 22 or 23 to measure collagen degradation in osteoarthritic cartilage.

25. A use of a kit according to claim 17, 18, 19, 20, 21, 22 or 23 to measure collagen degradation in intervertebral discs.

26. A monoclonal antibody, characterized by having the ability to bind to an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen.

27. A monoclonal antibody according to claim 26, wherein said monoclonal antibody has the ability to bind to an epitope

on unwound type II collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical type II collagen.

28. A monoclonal antibody according to claim 26, wherein said monoclonal antibody binds to a peptide containing an epitope having the following sequence (SEQ ID NO:4):

A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-.

29. The monoclonal antibody COL2-3/4m.

30. A cell line producing a monoclonal antibody which has the ability to bind to an epitope on unwound collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical collagen.

31. A cell line according to claim 30 wherein the monoclonal antibody produced has the ability to bind to an epitope on unwound type II collagen chains or fragments thereof containing said epitope, wherein said monoclonal antibody does not bind to native helical type II collagen.

32. A cell line according to claim 30, which has the identifying characteristics of ATCC HB 11202.

33. A synthetic peptide sequence characterized in that:
- it comprises at least a portion of a hydrophilic domain of peptide CB11 of Type II collagen α-chain; and
- it does not include hydroxylysine residues.

34. A synthetic peptide according to claim 33, characterized in that it comprises the following amino acid sequence (SEQ ID NO:4):

A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-.

35. A synthetic peptide according to claim 34, characterized in that it comprises the following amino acid sequence (residues 22-2 of SEQ ID NO:3):

G-K-V-G-P-S-G-A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-Q.

```
 N-1-   MIRLGAPQSL VLLTLLVAAV LRCQ                                    -SIGNAL P.
                                GQDVRQ PGPKGQKGEP GDIKDIVGPK          -N-PRO-P
  51-   GPPGPQGPAG EQGPRGDRGD KGEKGAPGPR GRDGEPGTLG NPGPPGPPGP
 101-   PGPPGLGGNF AA        CLINK  STR  PEP  STR
                   QMAGGFDE KAGGA↓QL↓GV↓M Q                          -N-TELO-P.
                                          GPMGPMGPR GPPGPAGAPG       -TRIP. HEL.
 151-   PQGFQGNPGE PGEPGVSGPM GPRGPPGPPG KPGDDGEAGK PGKAGERGPP       CNBr:1/2/3
 201-   GPQGARGFPG TPGLPGVKGH RGYPGLDGAK GEAGAPGVKG ESGSPGENGS       4/6/12
 251-   PGPM
            GPRGLP GERGRTGPAG AAGARGNDGQ PGPAGPPGPV GPAGGPGFPG       -TRIP. HEL.
 301-   APGAKGEAGP TGARGPEGAQ GPRGEPGTPG SPGPAGASGN PGTDGIPGAK       CNBr:11
 351-   GSAGAPGIAG APGFPGPRGP PDPQGATGPL GPKGQTGKPG IAGFKGEQGP
 401-   KGEPGPAGPQ GAPGPAGEEG KRGARGEPGG VGPIGPPGER GAPGNRGFPG
 451-   QDGLAGPKGA PGERGPSGLA GPKGANGDPG RPGEPGLPGA RGLTGRPGDA
 501-   GPQGKVGPSG APGEDGRPGP PGPQGARGQP GVM
                        CB11B                GFPGPKG ANGEPGKAGE       -TRIP. HEL.
 551-   KGLPGAPGLR GLPGKDGETG AEGPPGPAGP AGERGEQGAP GPSGFQGLPG       CNBr:8
 601-   PPGPPGEGGK PGDQGVPGEA GAPGLVGPRG ERGFPGERGS PGAQGLQGPR
 651-   GLPGTPGTDG PKGASGPAGP PGAQGPPGLQ GM
                                          PGERGAAG IAGPKGDRGD       -TRIP. HEL.
 701-   VGEKGPEGAP GKDGGRGLTG PIGPPGPAGA NGEKGEVGPP GPAGSAGARG       CNBr:10
 751-   APGERGETGP PGTSGIAGPP GADGQPGAKG EQGEAGQKGD AGAPGPQGPS
 801-   GAPGPQGPTG VTGPKGARGA QGPPGATGFP GAAGRVGPPG SNGNPGPPGP
 851-   PGPSGKDGPK GARGDSGPPG RAGEPGLQGP AGPPGEKGEP GDDGPSGAEG
 901-   PPGPQGLAGQ RGIVGLPGQR GERGFPGLPG PSGEPGQQGA PGASGDRGPP
 951-   GPVGPPGLTG PAGEPGREGS PGADGPPGRD GAAGVKGDRG ETGAVGAPGA
1001-   PGPPGSPGPA GPTGKQGDRG EAGAQGPM
                                      GP SGPAGARGIQ GPQGPRGDKG       -TRIP. HEL.
1051-   EAGEPGERGL KGHRGFTGLQ GLPGPPGPSG DQGASGPAGP SGPRGPPGPV       CNBr:5/9/7
1101-   GPSGKDGAMG IPGPIGPPGP RGRSGETGPA GPPGNPGPPG PPGPP            14/15
                                                      GPGID         -C-TELO-P.
1151-   MSAFAGLGPR EKGPDPLQYM RA
                             DQAAGGLR QHDAEVDATL KSLNNQIESI         -C-PRO-P.
1201-   RSPEGSRKNP ARTCRDLKLC HPEWKSGDYW IDPNQGCTLD AMKVFCNMET
1251-   GETCVYPNPA NVPKKNWWSS KSKEKKHIWF GETINGGFHF SYGDDNLAPN
1301-   TANVQMTFLR LLSTEGSQNI TYHCKNCIAY LDEAAGNLKK ALLIQGSNDV
1351-   EIRAEGNSRF TYTALKDGCT KHTGKWGKTV IEYRSQKTSR LPIIDIAPMD
1401-   IGGPEQEFGV DIGPVCFL                                         -C
```

FIG. 1

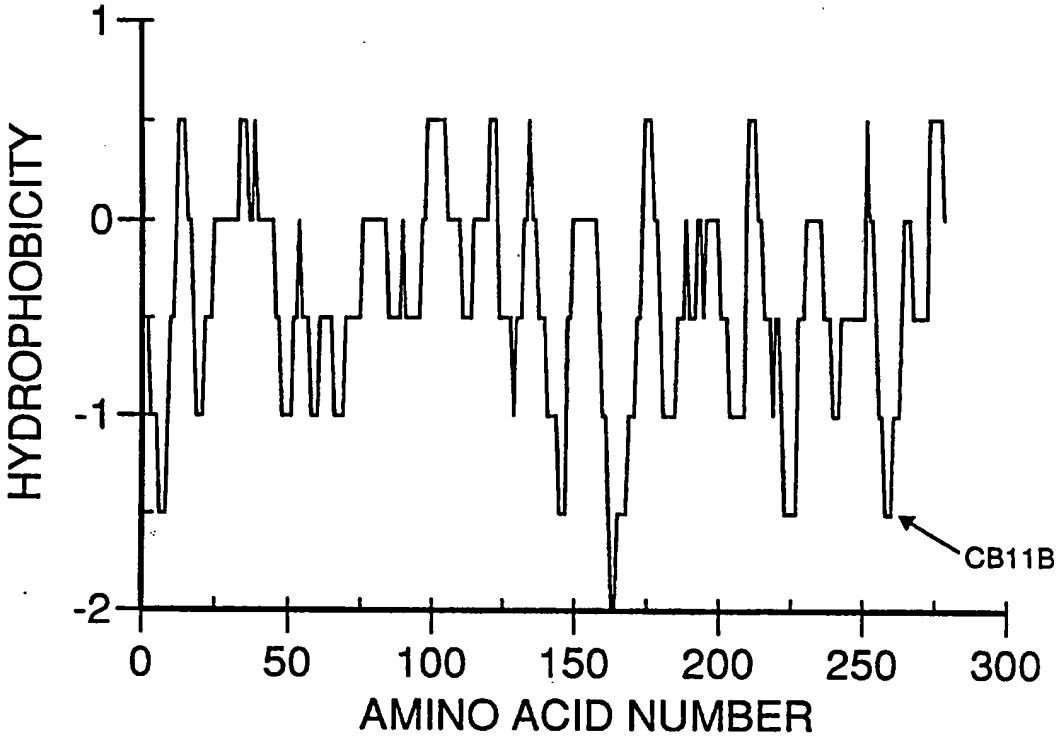
1
0
-1
-2
HYDROPHOBICITY
0
50
100
150
200
250
300
AMINO ACID NUMBER
CB11B

FIG. 2

A) **CB11B**

C-G-K-V-G-P-S-G-A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-Q-Y

B) **CB11B/H**

-A-P(OH)-G-E-D-G-R-P(OH)-G-P-P(OH)-G-P-

FIG. 3

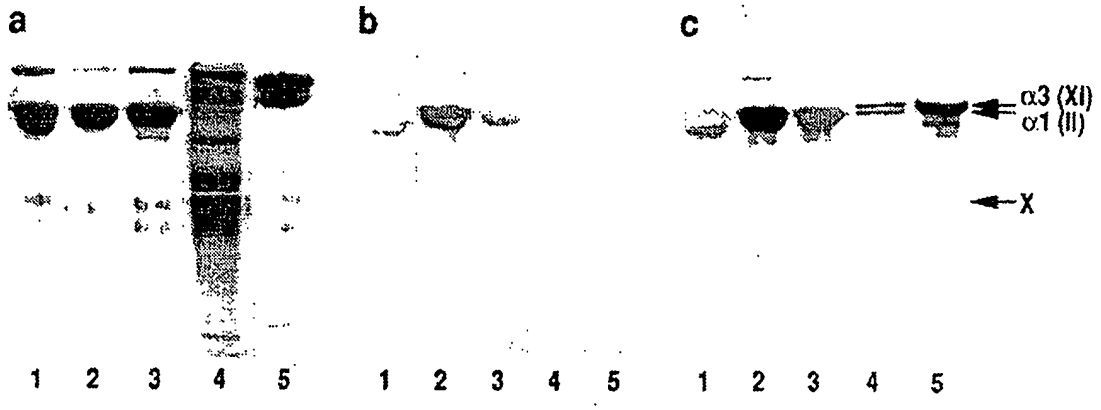

FIG. 4

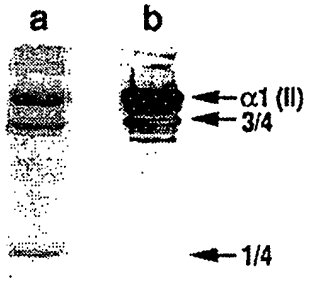

FIG. 5

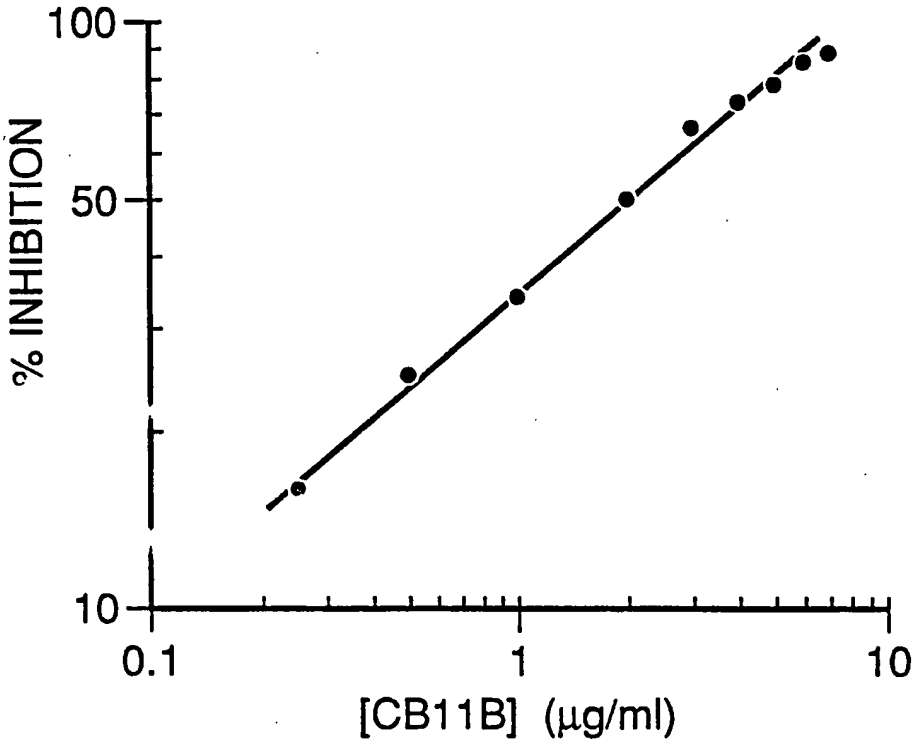

FIG. 6

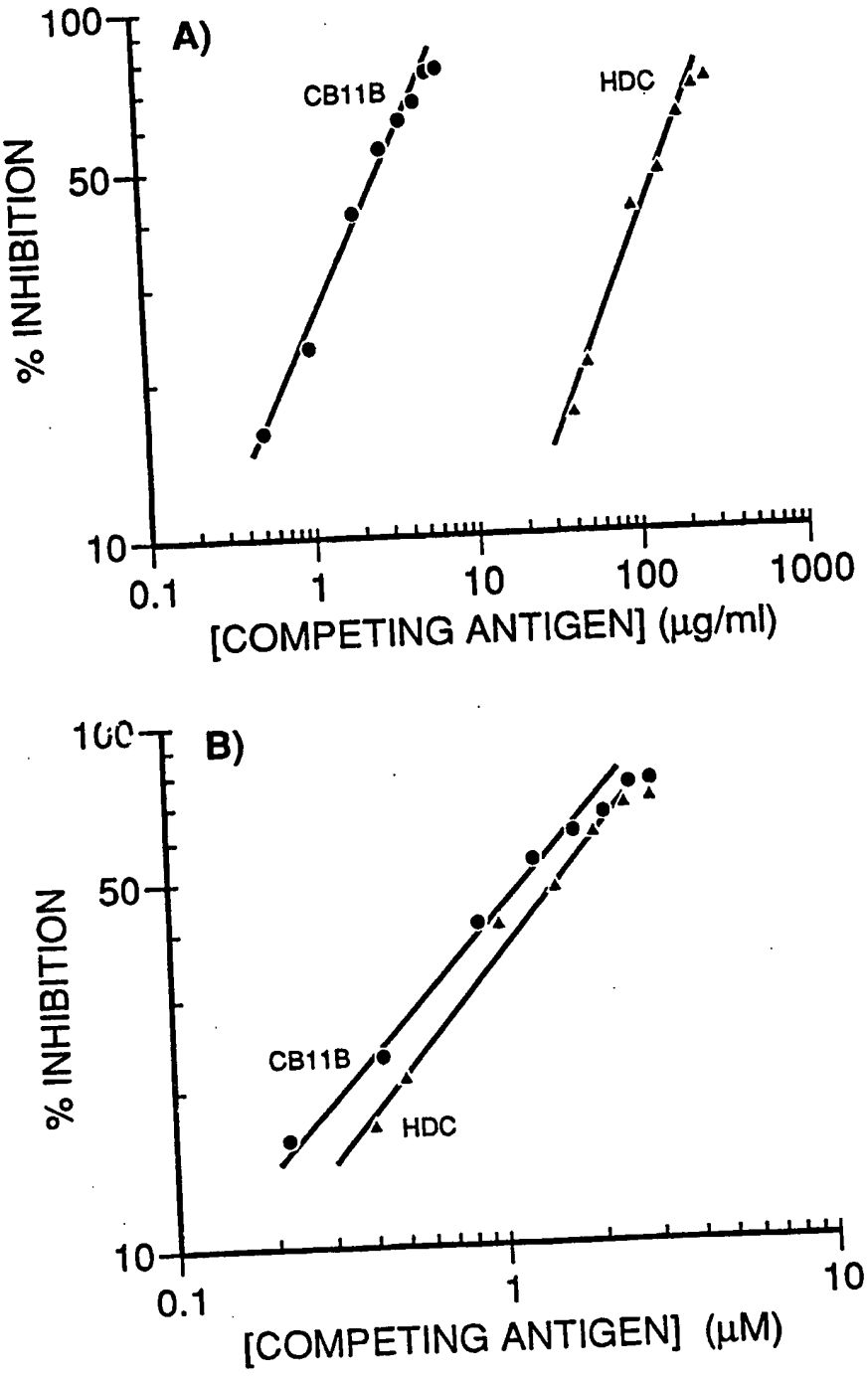
A)
100
50
10
% INHIBITION
CB11B
HDC
0.1
1
10
100
1000
[COMPETING ANTIGEN] (µg/ml)
B)
100
50
10
% INHIBITION
CB11B
HDC
0.1
1
10
[COMPETING ANTIGEN] (µM)

FIG. 7

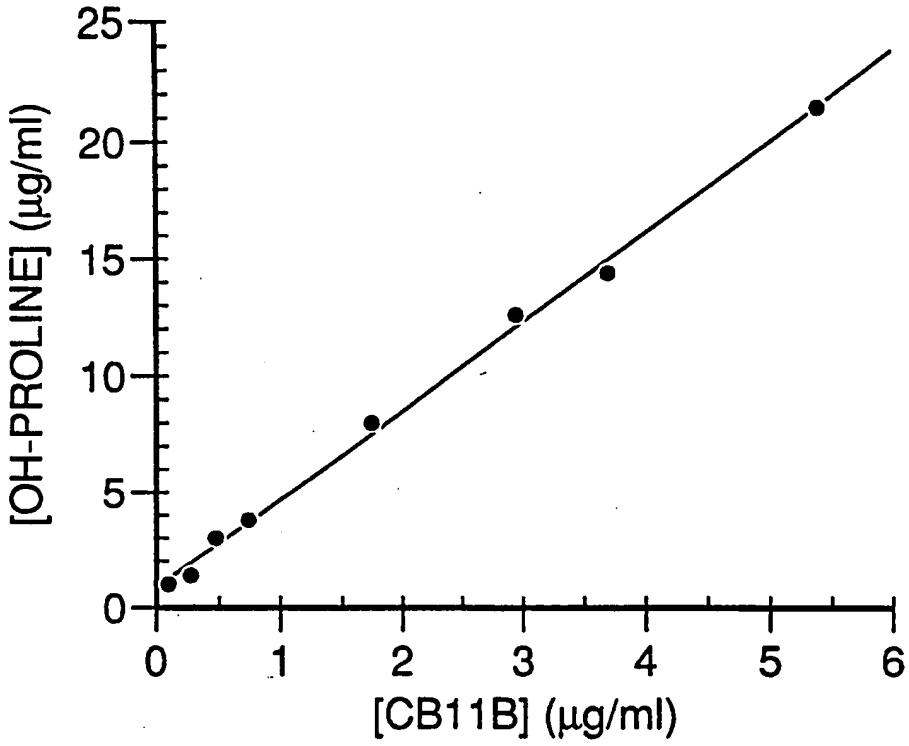

FIG. 8

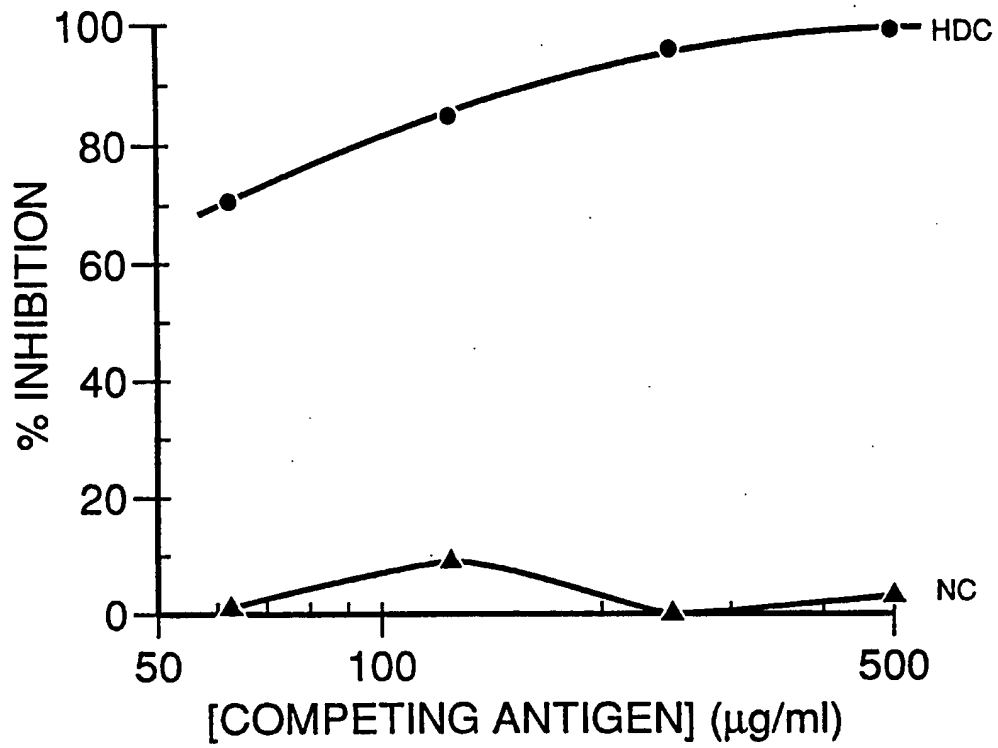
100
80
60
40
20
0
% INHIBITION
HDC
NC
50
100
500
[COMPETING ANTIGEN] (µg/ml)

FIG. 9

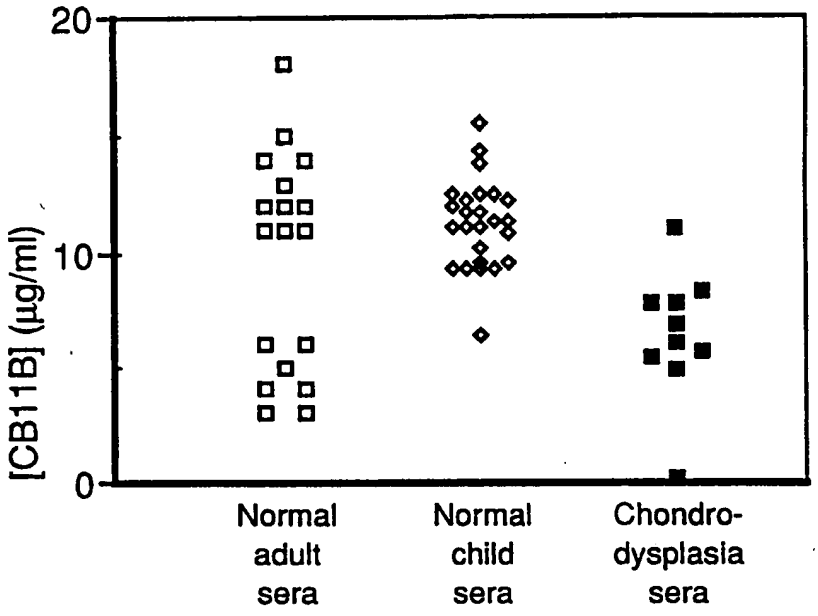
20
10
0
[CB11B] (µg/ml)
Normal adult sera
Normal child sera
Chondro-dysplasia sera

FIG. 10

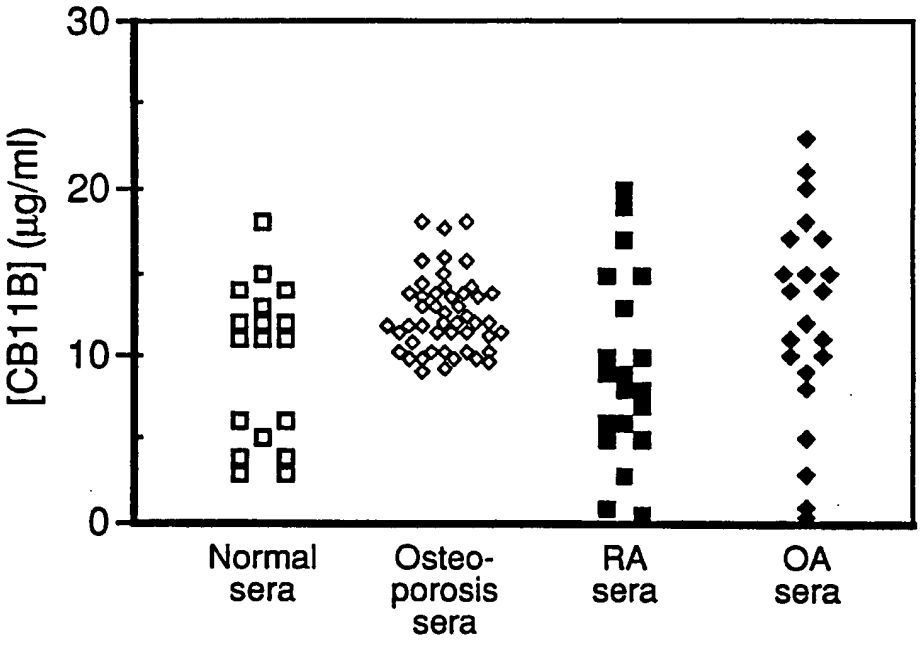
30
20
10
0
[CB11B] (µg/ml)
Normal sera
Osteo-porosis sera
RA sera
OA sera

FIG. 11

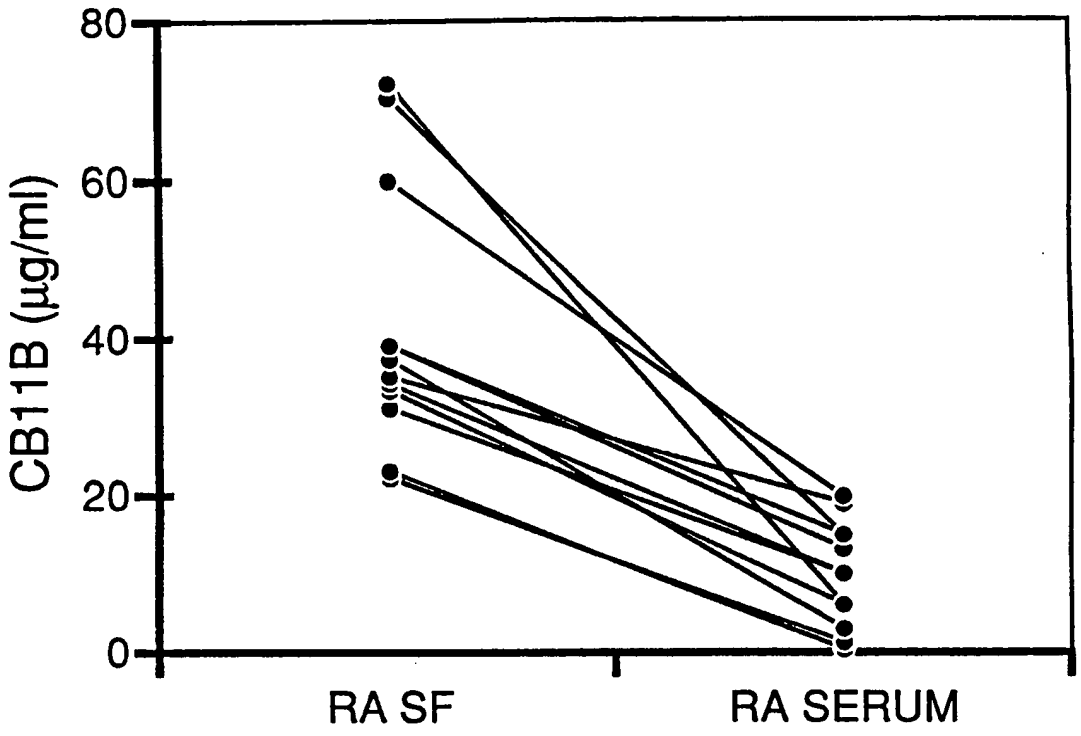

FIG.12

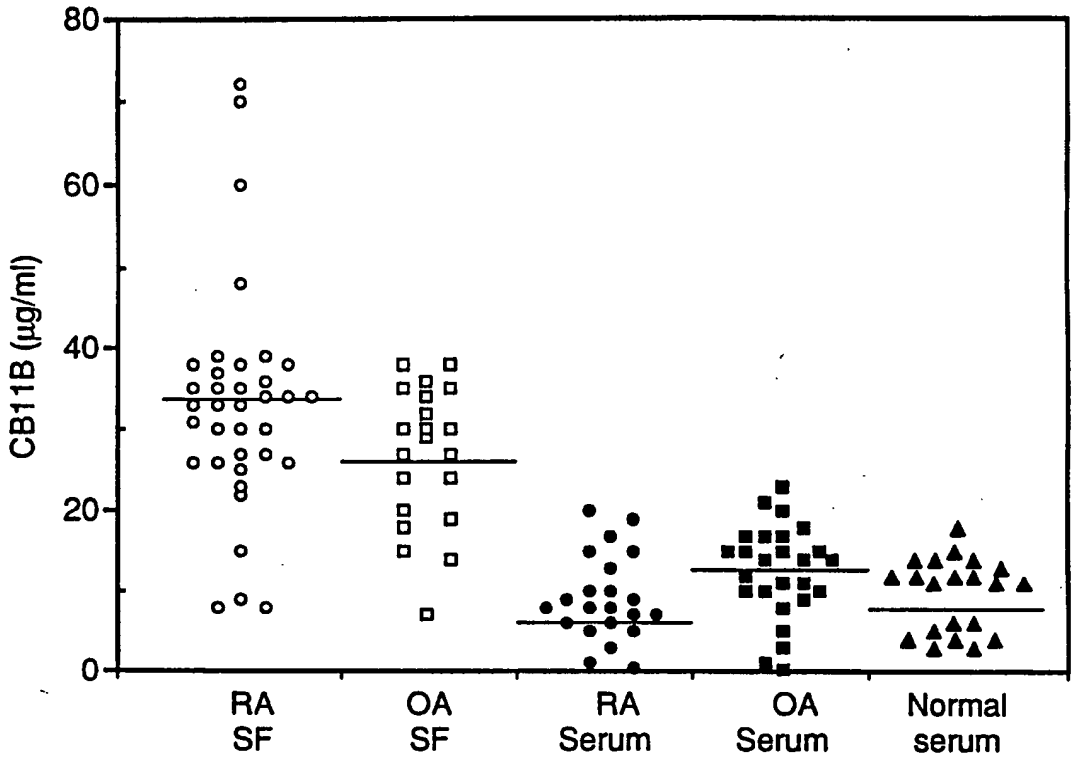
80
60
40
20
0
CB11B (µg/ml)
RA SF
OA SF
RA Serum
OA Serum
Normal serum

FIG. 13

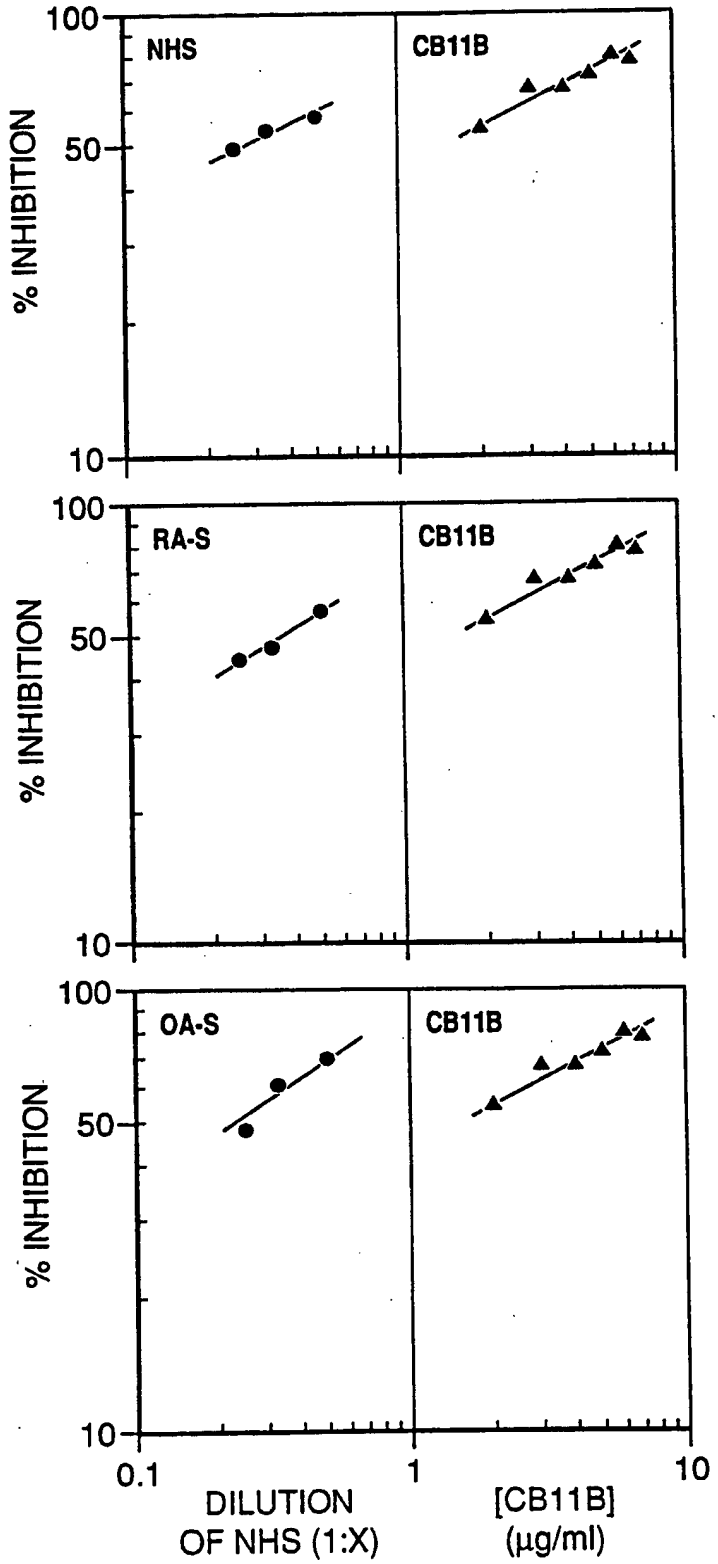
100
50
10
% INHIBITION
NHS
CB11B
RA-S
OA-S
0.1
1
10
DILUTION
OF NHS (1:X)
[CB11B]
(µg/ml)

FIG. 14

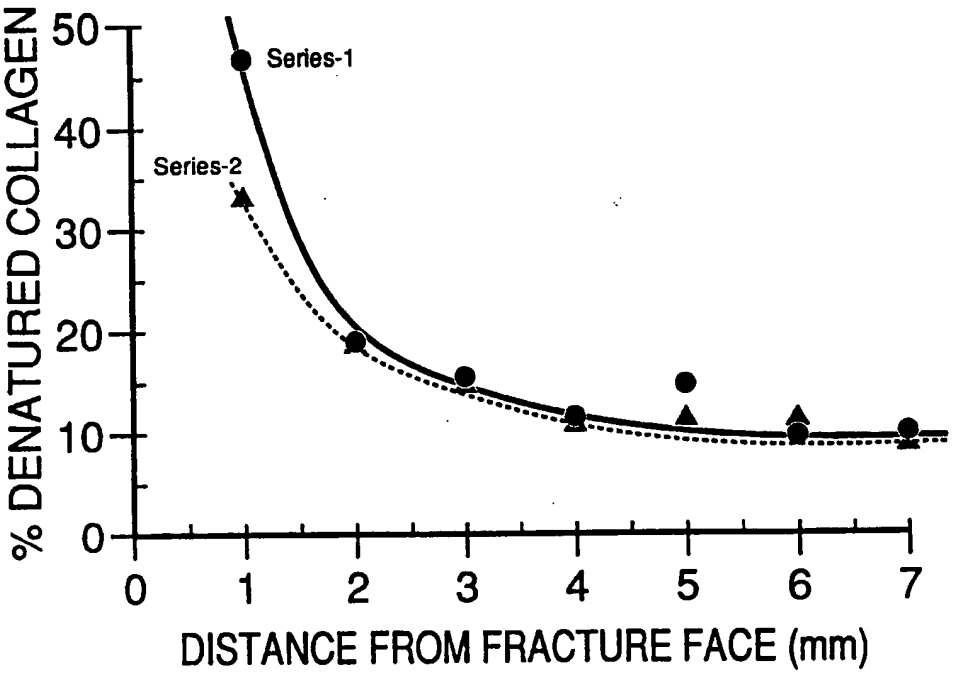
% DENATURED COLLAGEN
50
40
30
20
10
0
Series-1
Series-2
0
1
2
3
4
5
6
7
DISTANCE FROM FRACTURE FACE (mm)

FIG. 15

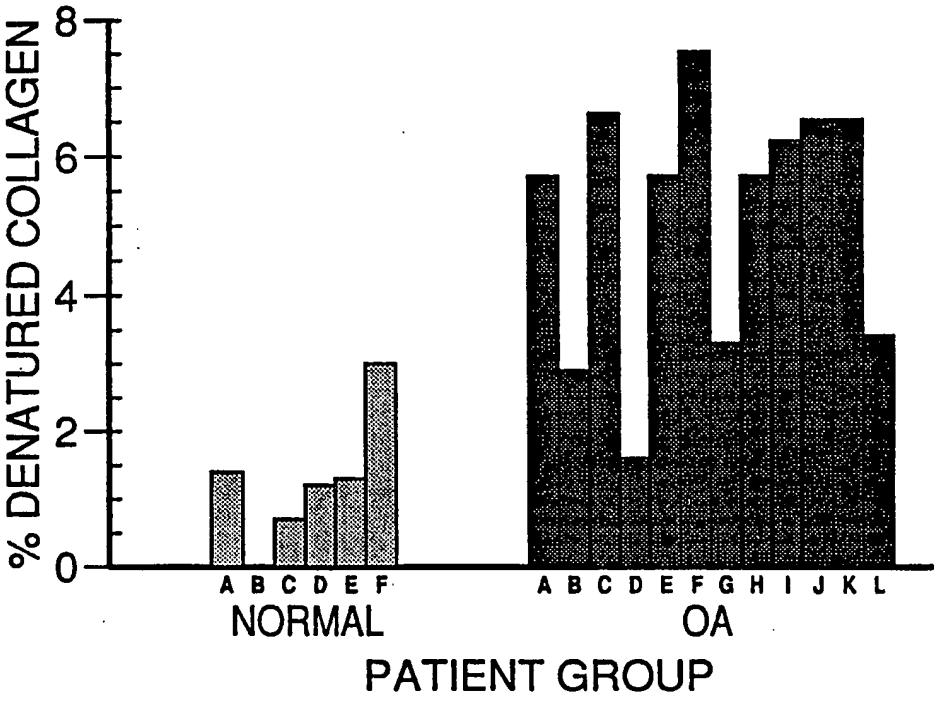
% DENATURED COLLAGEN
0
2
4
6
8
A B C D E F
NORMAL
A B C D E F G H I J K L
OA
PATIENT GROUP

FIG.16

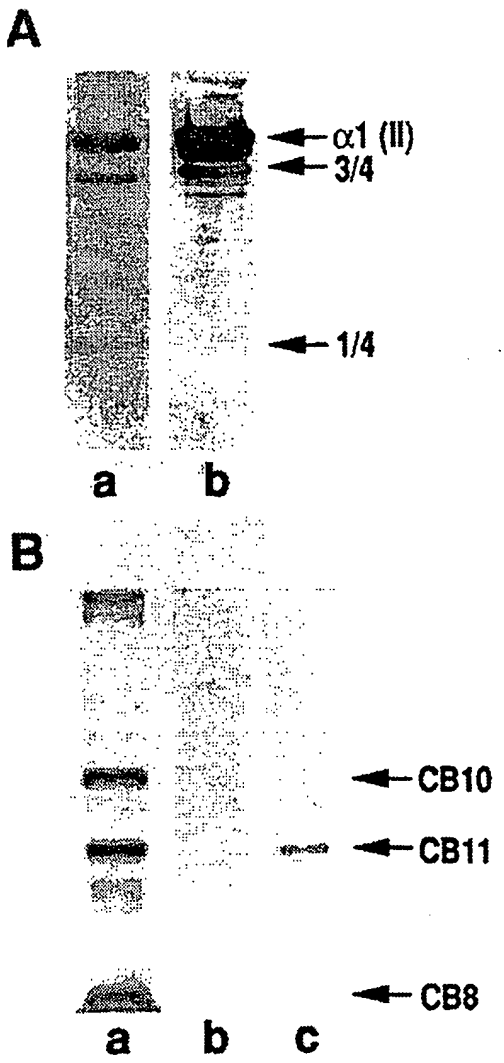

FIG. 17

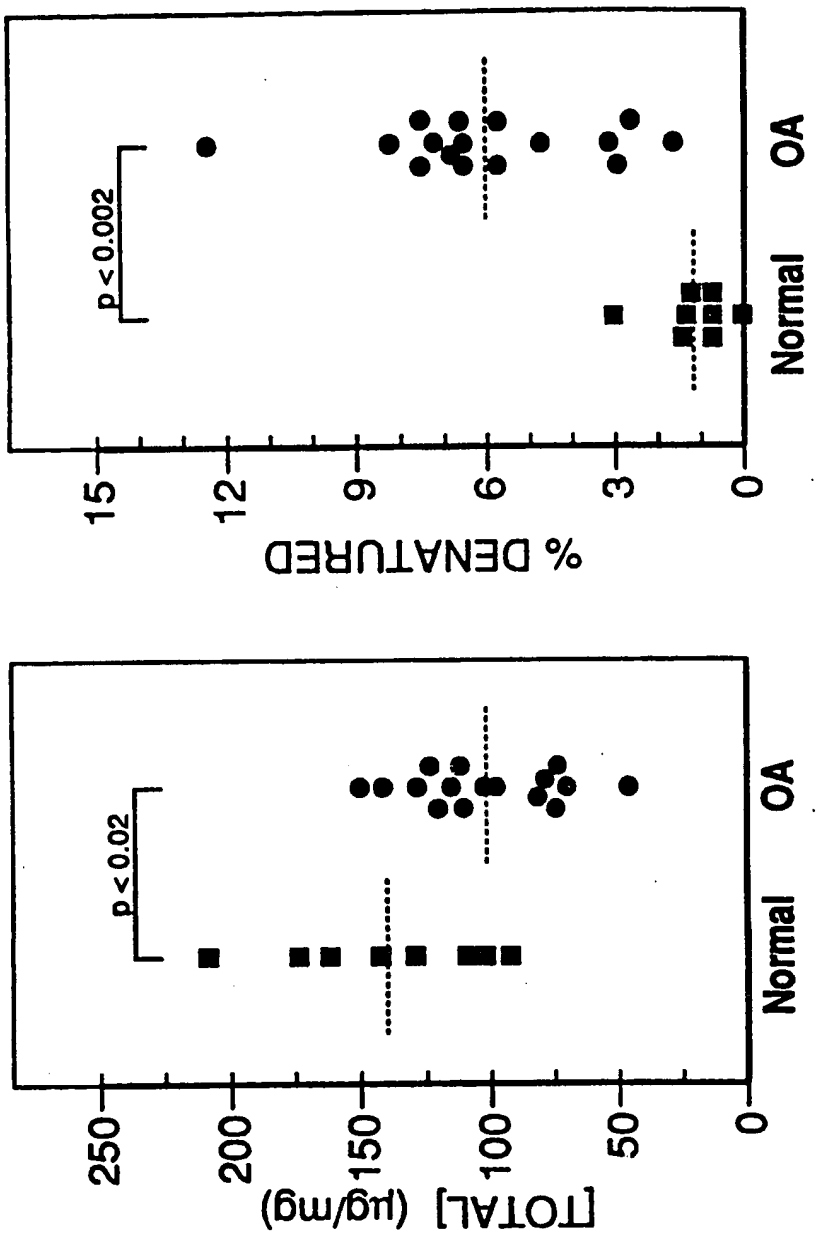
% DENATURED
15
12
9
6
3
0
p < 0.002
Normal
OA
[TOTAL] (µg/mg)
250
200
150
100
50
0
p < 0.02
Normal
OA

FIG. 18

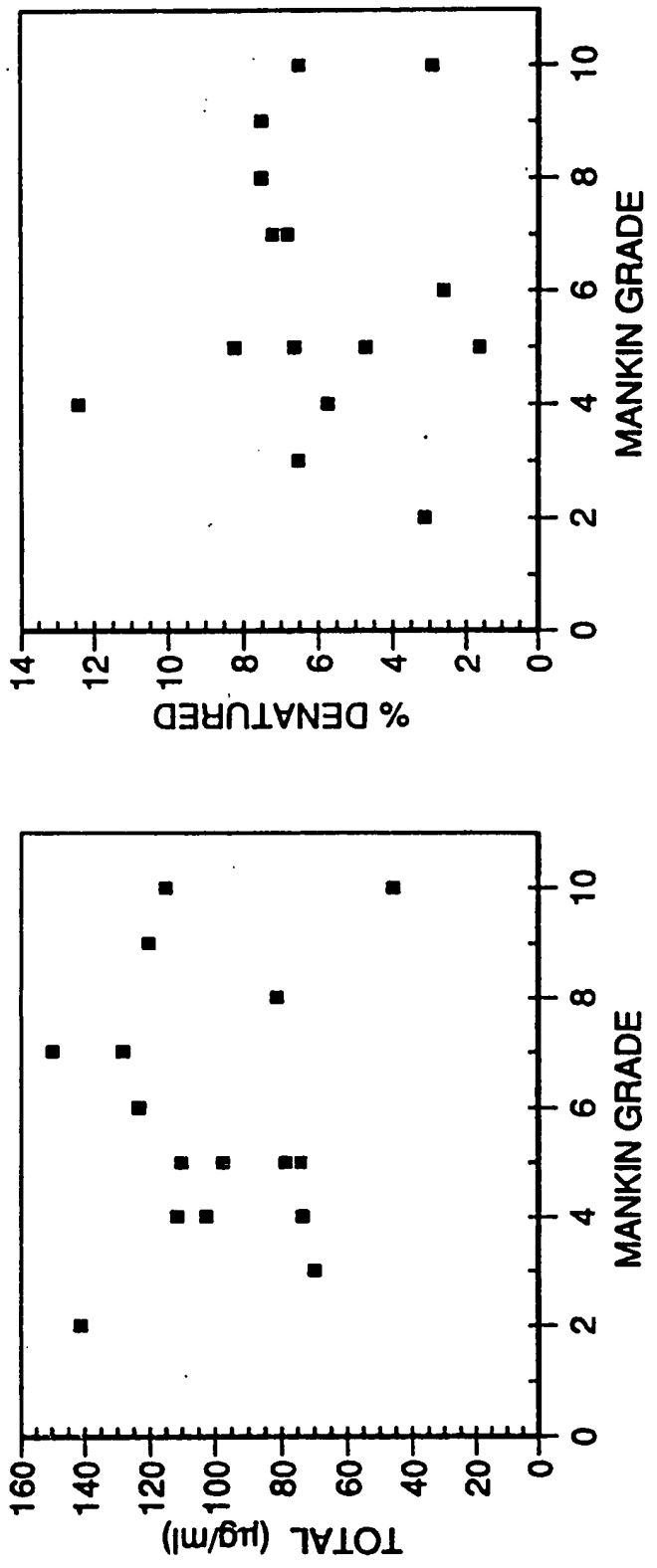
% DENATURED
0
2
4
6
8
10
12
14
MANKIN GRADE
0
2
4
6
8
10
TOTAL (µg/ml)
0
20
40
60
80
100
120
140
160
MANKIN GRADE
0
2
4
6
8
10

FIG. 19

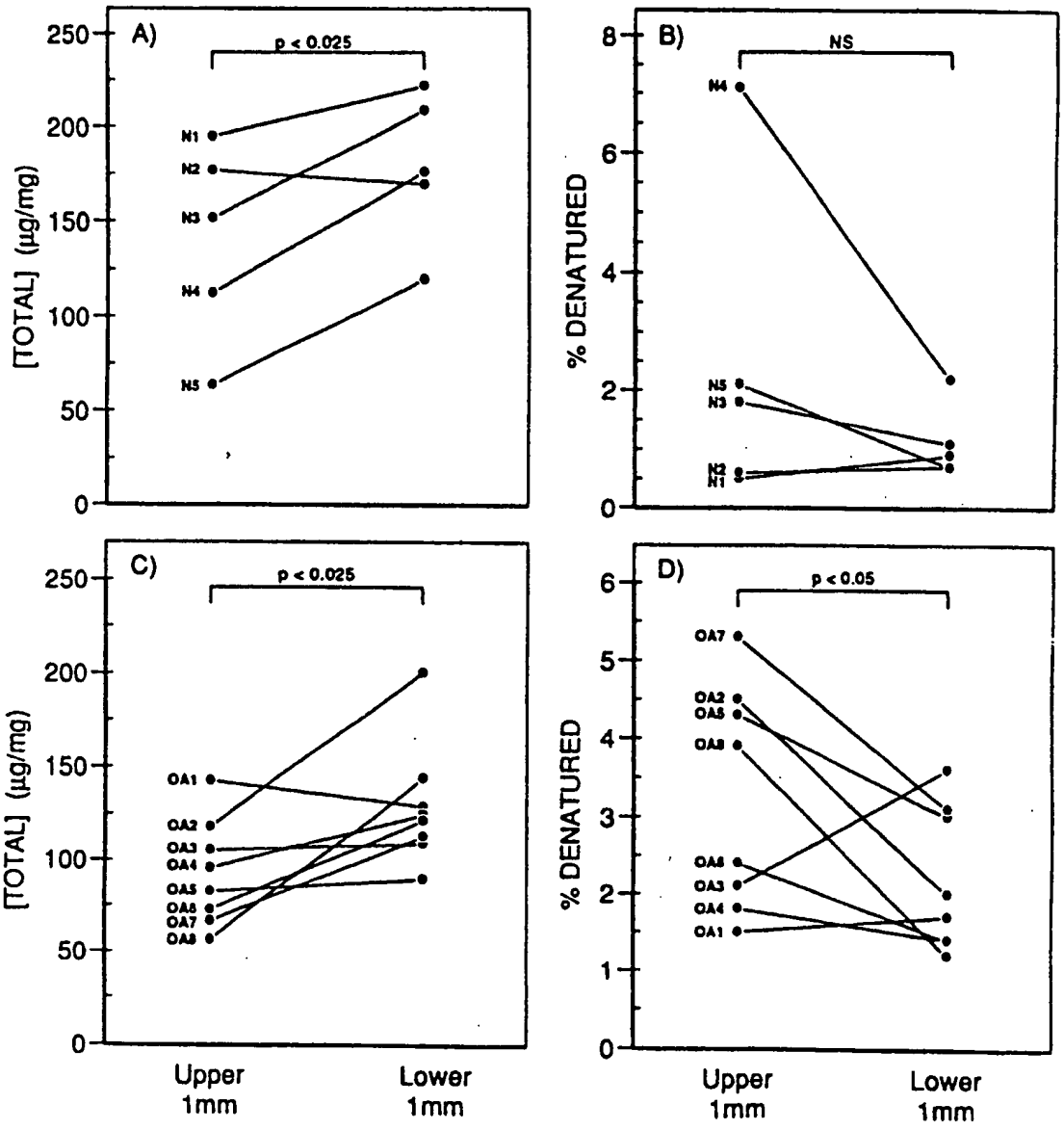
A)
p < 0.025
[TOTAL] (µg/mg)
250
200
150
100
50
0
N1
N2
N3
N4
N5
B)
NS
% DENATURED
8
6
4
2
0
C)
p < 0.025
[TOTAL] (µg/mg)
OA1
OA2
OA3
OA4
OA5
OA6
OA7
OA8
Upper 1mm
Lower 1mm
D)
p < 0.05
% DENATURED
6
5
4
3
2
1
0
OA7
OA2
OA5
OA8
OA6
OA3
OA4
OA1
Upper 1mm
Lower 1mm

FIG.20

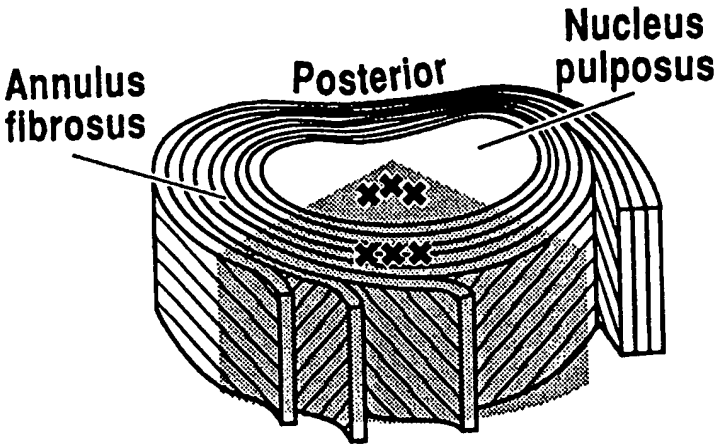

FIG. 21

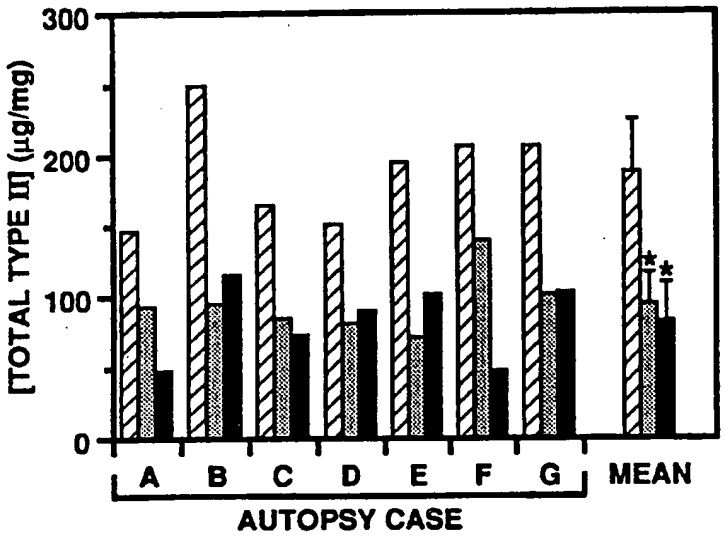

FIG. 22

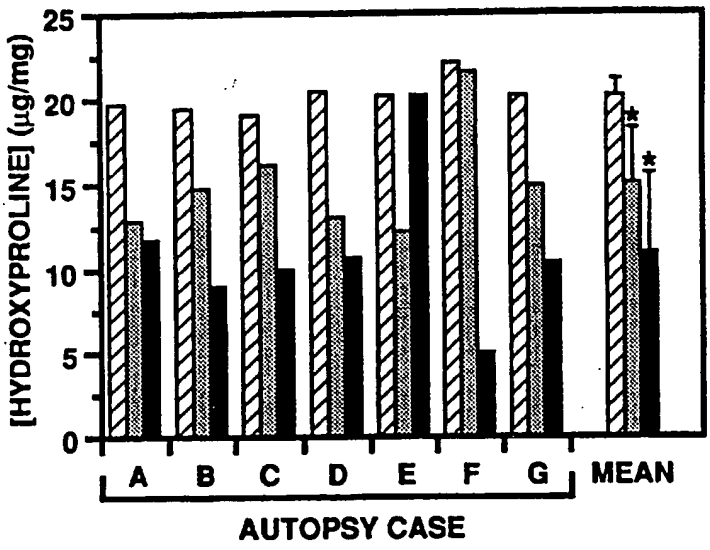
[HYDROXYPROLINE] (µg/mg)
25
20
15
10
5
0
A
B
C
D
E
F
G
MEAN
AUTOPSY CASE

FIG. 23

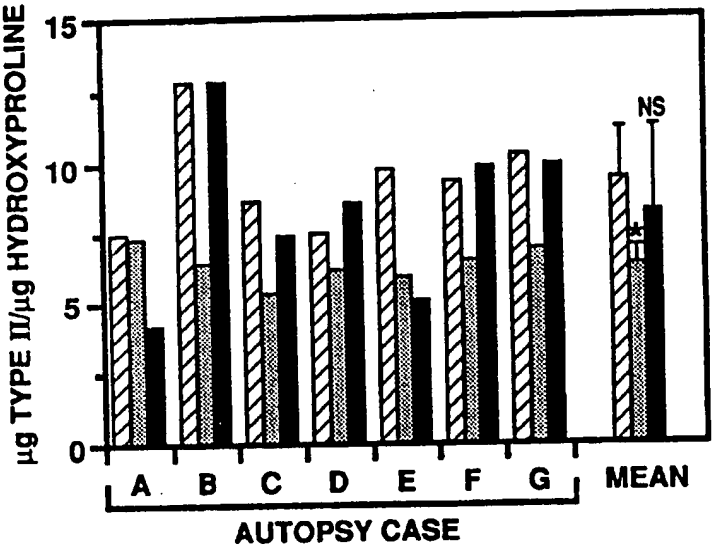
μg TYPE II/μg HYDROXYPROLINE
15
10
5
0
NS
*
A
B
C
D
E
F
G
MEAN
AUTOPSY CASE

FIG. 24

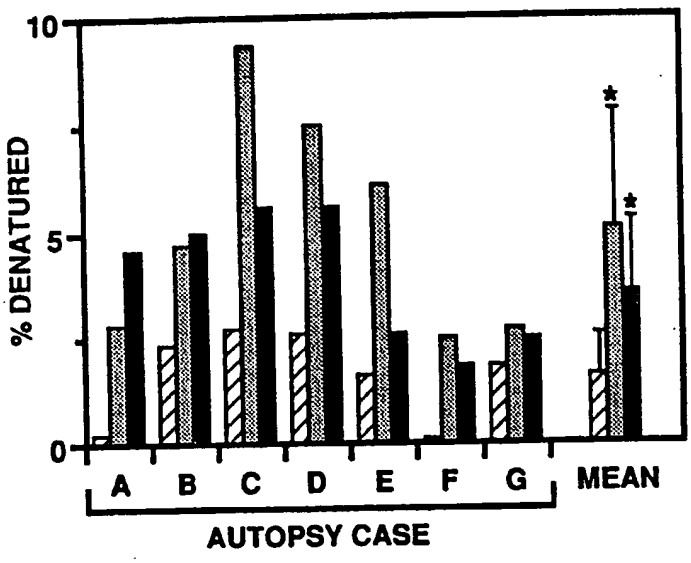

FIG. 25

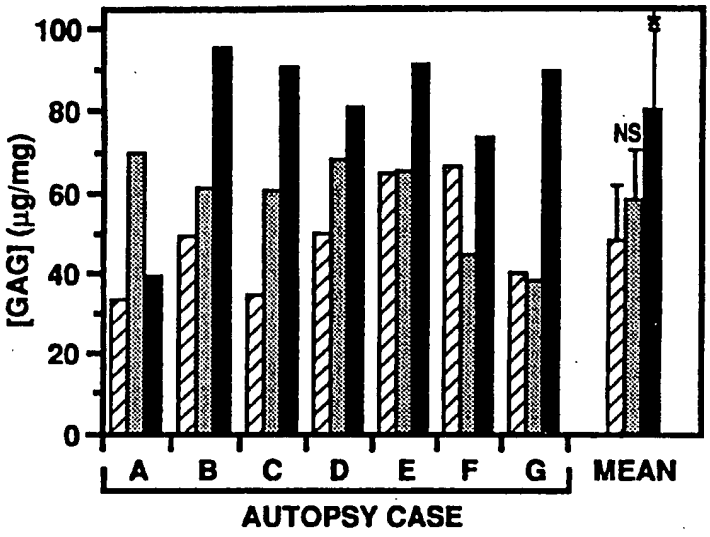

FIG. 26

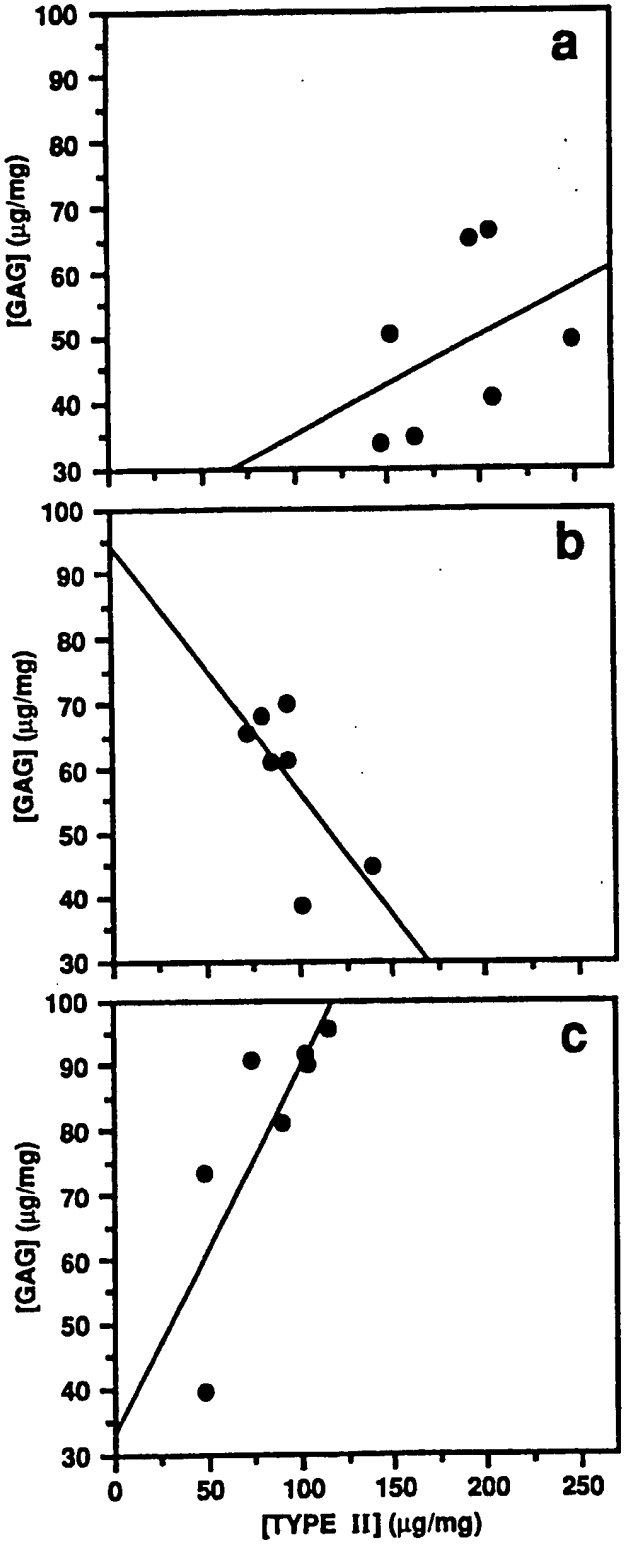
a
b
c
[GAG] (µg/mg)
[TYPE II] (µg/mg)
100
90
80
70
60
50
40
30
0
50
100
150
200
250

FIG. 27

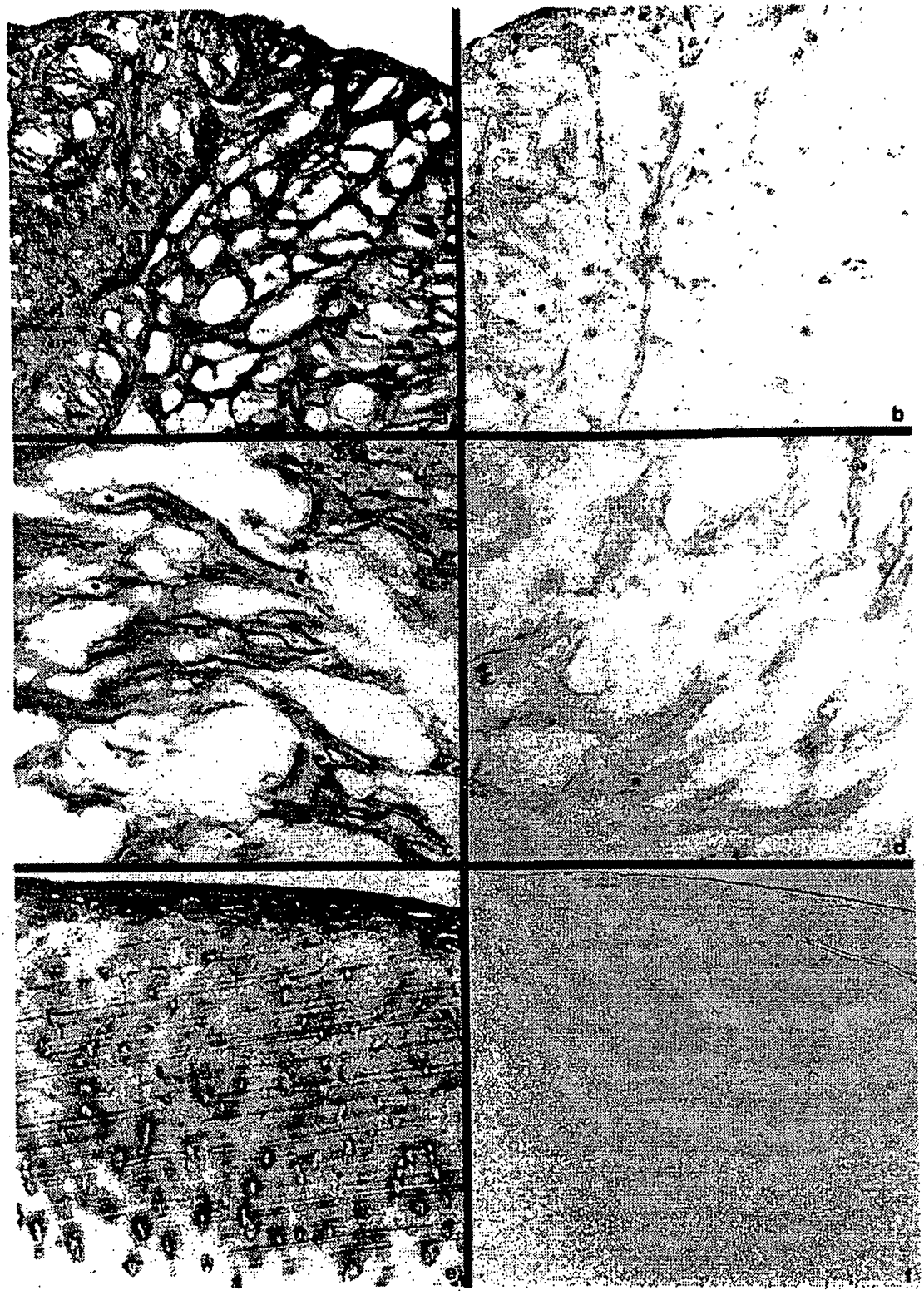

FIG 28

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/CA 93/00522

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/68 G01N33/577 C12P21/08 C12N5/18 C07K7/08
C07K7/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N C07K C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IMMUNOLOGY vol. 77 , 1992 , OXFORD, GB pages 609 - 616 K. MORGAN ET AL. 'Identification of an immunodominant B-cell epitope in bovine type II collagen and the production of antibodies to type II collagen by immunization with a synthetic peptide representing this epitope' see the whole document --- -/--	1-29, 33-35

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 March 1994	07. 04. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Doepfer, K-P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	MATRIX vol. 13, no. 2 , February 1993 , STUTTGART, DE pages 95 - 102 JOHN S. MORT ET AL. 'Direct Evidence for Active Metalloproteinases Mediating Matrix Degradation in Interleukin 1-sStimulated Human Articular Cartilage' cited in the application see page 97, left column, line 40 - right column, line 43	1-25
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 262, no. 23 , 15 August 1987 , BALTIMORE, MD US pages 11345 - 11350 NICHOLAS P. MORRIS AND HANS PETER BÄCHINGER 'Type XI Collagen Is a Heterotrimer with the Composition (1alpha,2alpha,3alpha) Retaining Non-trple-helical Domains' cited in the application see the whole document	1-25
A	THE JOURNAL OF CLINICAL INVESTIGATION vol. 83, no. 2 , February 1989 , NEW YORK, NY, US pages 647 - 661 GEORGE R. DODGE AND A. ROBIN POOLE 'Immunohistochemical Analysis of Type II Collagen Degradation in Human Normal, Rheumatoid, and Osteoarthritic Cartilages and in Explants of Bovine Articular Cartilage Cultured with Interleukin 1' cited in the application see the whole document	1-25
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 118, no. 3 , 14 February 1984 , DULUTH, MINNESOTA US pages 724 - 729 DAVID R. EYRE ET AL. 'All three chains of 1alpha2alpha3alpha collagen from hyaline cartilage resist human collagenase' cited in the application see the whole document	1-25
A	EP,A,0 505 210 (ORION-YHTYMÄ OY) 23 September 1992 see the whole document	1-29

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indicanon, where appropriate, of the relevant passages	Relevant to claim No.
A	SEMINARS IN ARTHRITIS AND RHEUMATISM vol. 13, no. 1 , August 1983 , NEW YORK, NY, US pages 1 - 86 MARCEL E. NIMNI 'Collagen: Structure, Function, and Metabolism in Normal and Fibrotic Tissues' cited in the application see page 14, left column, line 4 - page 17, left column, line 45 see page 25, left column, line 33 - page 27, left column, line 34	1-25

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Interr al Application No
PCT/CA 93/00522

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0505210	23-09-92	NONE	